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Predation of cyanobacteria by Acanthamoeba SPP

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PREDATION OF CYANOBACTERIA BY ACANTHAMOEBA SPP.

Submitted by Robert Cumming Dryden

for the degree of Ph.D.

of the University of Bath

1987

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SUMMARY

Strains of Acanthamoeba (principally A. castellanii and A. polyphaga) were found to predate a wide range of unicellular and filamentous cyanobacteria in both lawn and liquid culture. The resistance to predation of some cyanobacterial species was attributed to the production of copious mucilage. Predation of cyanobacteria led to the proliferation of amoebae at the expense of the cyanobacterial population. Exhaustion of the cyanobacterial food source led to encystment of Acanthamoeba.

The rate of ingestion of Anabaena by A. castellanii was strongly affected by temperature, being minimal at 4°C and maximal at 20°C and 30°C. Ingestion rate in turn affected the rate of amoebal proliferation, which was greatest at 20°C. Quantitative differences were found in the ability of different cyanobacterial species to support amoebal proliferation.

In illuminated batch cultures Acanthamoeba eliminated populations of Anabaena but not those of Synechococcus. This variation in effectiveness of predation was attributable to differences in the size, growth form and reproductive rate of the prey. Anabaena populations were eliminated at all temperatures (15°C, 22.5°C & 30°C) despite incubation in conditions which promoted cyanobacterial growth. The rate of clearance of Anabaena was dependent on the size of the amoebal inoculum. Clearance of cyanobacteria did not occur when amoebal inocula were below a critical size.

Thin-section transmission electron microscopy of feeding trophozoites revealed that ingested cyanobacteria underwent

progressive digestion in food vacuoles.

The presence of lysozyme-like enzymes was clearly demonstrated in crude extracts prepared from amoebal trophozoites. Addition of extracts to cyanobacterial suspensions led to morphological disruption of cyanobacterial cells.

INTRODUCTION AND LITERATURE REVIEW

Protozoa and cyanobacteria: Introduction, definition and ecology

Protozoa

Protozoa may be defined as unicellular eukaryotes (protists) in which the dominant form of nutrition is heterotrophy. They can best be considered as a Sub-kingdom of the Kingdom Protista. They are not a natural grouping, the only common relationship being their unicellular organization, but are placed together for convenience. Protozoa are almost unparalleled in diversity of structure and function, and are capable of establishing themselves in almost every conceivable habitat provided it contains a suitable food source and moisture in some form. Most species are between 5 and 250 μm in size. At present seven "protozoan" phyla are recognized (Levine et al., 1980), although several of these are certainly polyphyletic. Approximately 30 000 extant species have been described, including 11 550 sarcodines, 6 900 flagellates and 7 200 ciliates (Levine et al., 1980). Although the great majority of species are heterotrophic, a number of predominantly photosynthetic flagellate groups (e.g. Chrysomonadida, Euglenida) are included because they contain heterotrophic representatives.

Protozoa adopt a number of ecological roles. Certain flagellate groups (particularly dinoflagellates) are important as primary-producers in aquatic habitats. Ciliate and heterotrophic flagellate protozoa within the freshwater plankton are important grazers of bacterial populations, functioning as intermediaries transforming fine organic matter into a particle size range readily available to crustacean zooplankters (Porter, Pace & Battey, 1979;

Porter et al., 1985). In this way the energy of decomposers is returned to higher trophic levels.

Protozoa are also of central importance in controlling bacterial numbers in soils. Clarholm (1981) recorded a 20-fold increase in amoebal numbers as a response to rises in bacterial populations in the four days following rain. Stout & Heal (1967) calculated that in arable fields protozoa consumed 150-900g bacteria $m^{-2}year^{-1}$. In contrast to aquatic habitats most predation in soil is by amoebae, whose small size enables penetration of pore spaces and movement in the water films surrounding soil particles (Clarholm, 1981). By their consumption of bacteria soil protozoa release nutrients which are then available for uptake by plants, so enhancing the fertility of the soil (Bamforth, 1985).

In marine food chains the ingestion of unicellular cyanobacteria by phagotrophic protozoa (mainly small flagellates and ciliates) is an important link between primary-producers and multicellular copepod consumers (Johnson, Xu & Sieburth, 1982). Protozoan predation of chemoorganotrophic bacteria (which utilize production "lost" through dissolved organic matter release) may represent an efficient pathway of nutrient recovery in marine ecosystems.

Cyanobacteria

The cyanobacteria (formerly known as blue-green algae) are a unique group of organisms in possessing a prokaryotic cellular organization in combination with oxygen-evolving photosynthesis similar to that of eukaryotic algae and higher plants. They contain

chlorophyll a, and also form phycobilin-protein pigment complexes. Photoautotrophy is the predominant form of nutrition, although some species can live photoheterotrophically in dim light and others heterotrophically in the dark. Many species are capable of nitrogen fixation, usually in specialized cells known as heterocysts. Cyanobacteria are one of the most diverse prokaryotic groups in terms of the number and variety of species. Most cyanobacterial cells are 2-5 μm in diameter, and are found in a range of growth forms including minute single cells, simple filaments, coils and branched multiseriate filaments. Some species may form rafts or mats of indeterminate size and shape.

The taxonomy of cyanobacteria has been and still is a source of much confusion and debate. Whilst traditionally classified under the provisions of the botanical code (resulting from their treatment as algae), Stanier et al. (1978) submitted a proposal which would bring them under the rules of the bacteriological code. Rippka et al. (1979) have published revised definitions of many cyanobacterial genera, placing them in five sections each distinguished by a particular pattern of structure and development. In this thesis the traditional phycological nomenclature has been retained since many of the references antedate 1979, and because in many cases the descriptions given in the original publications are inadequate to allow reclassification to a currently recognized taxon.

Cyanobacteria are of worldwide occurrence, with many species cosmopolitan in distribution. Ecologically they are of great importance both as primary producers (where they form the basis of many food chains) and as nitrogen fixers. Nitrogen fixation by desert

algal crusts probably provides a major input of nitrogen into desert ecosystems (Fuller, Cameron & Raica, 1960), and cyanobacterial nitrogen fixation is also paramount to the fertility of soil in rice fields. Cyanobacteria proliferate in eutrophic lakes of frigid, temperate and tropical regions, and vast populations of the filamentous Trichodesmium may develop in tropical seas. Unicellular cyanobacteria within the picoplankton are a ubiquitous and conspicuous component of both coastal and oceanic waters (Johnson & Sieburth, 1979). The resistance of many cyanobacteria to high temperatures makes them important colonizers of thermal waters.

In recent years there has been a marked upsurge in interest in cyanobacteria due to their environmental importance, their potential as a food source, and their suitability as model systems for fundamental physiological and genetic research (Carr & Whitton, 1982).

The taxonomy and distribution of Acanthamoeba

Taxonomy

Acanthamoebae are small rhizopod amoebae belonging to the order Amoebida. Acanthamoeba is the only genus within the family Acanthamoebidae (see Table 1.1 for formal classification). Cells of Acanthamoeba occur as two interchangeable forms, a locomotory feeding stage (trophozoite) and a resting stage (cyst). Trophozoites characteristically have a broad hyaline zone bearing many slender, tapering, flexible pseudopodia (acanthopodia). Cysts are polyhedral or thickly biconvex, with a cellulose-containing wall composed of two layers ; a more or less polygonal or stellate endocyst and a more or

less rippled ectocyst. Excystment is by removal of an operculum at a point of contact between endocyst and ectocyst (Page, 1976). Reproduction of acanthamoebae is by binary fission. The form of the pseudopodia and cysts distinguishes Acanthamoeba from the genera Hartmannella and Mayorella, with which there has been much past confusion.

Table 1.1 The Classification of Acanthamoeba (from Lee, Hutner & Bovee, 1985).

Phylum	:	Sarcomastigophora
Subphylum	:	Sarcodina
Superclass	:	Rhizopoda
Class	:	Lobosea
Subclass	:	Gymnamoebia
Order	:	Amoebida
Suborder	:	Acanthopodina
Family	:	Acanthamoebidae
Genus	:	Acanthamoeba

Whilst the genus is relatively distinct, the characteristics of individual species are not well clarified, with much overlapping. This is mainly due to the lack of stable morphological features with which to compare isolates. Page (1967) describes seven species from soil and freshwater on the basis of cyst structure : A. astronyxis, A. comandoni, A. palestinensis, A. culbertsoni, A. griffini, A. castellanii and A. polyphaga. Pussard & Pons (1977) proposed eight new

species as a result of light microscopic observations on cyst wall morphology. A number of other species have been described, including A. tubiashi (Lewis & Sawyer, 1979) and A. royreba (Willaert, Stevens & Tyndall, 1977).

The problem of resolving the intrageneric status of strains by morphological markers has increasingly led workers to explore other techniques as aids to taxonomy. The necessity for rapid, accurate identification of Acanthamoeba isolates is particularly pressing in view of the potential pathogenicity of some strains (see below). Techniques employed include mitochondrial DNA variation (Costas et al., 1983 ; Byers, Bogler & Burianek, 1983), starch-gel electrophoresis of esterases and acid phosphatases (Costas & Griffiths, 1980) and fatty acid composition (Costas & Griffiths, 1984). Information on taxonomic groupings can also be gained by comparing the enzyme activities of Acanthamoeba strains against a range of substrates (Costas & Griffiths, 1985).

Acanthamoeba castellanii is generally recognized as the type species. Initially reported by Castellani (1930a) as a contaminant in cultures of the yeast Cryptococcus pararoseus, it was subsequently described as a new species of Hartmannella, H. castellanii (Douglas, 1930). The species was transferred to the newly-erected genus Acanthamoeba by Volkonsky (1931).

Numbers and distribution

Amoebae of the genus Acanthamoeba are among the most common of all protozoa (Page, 1976). Most species have a very wide geographical distribution, occurring almost anywhere within the broad dictates of

their ecological requirements, foremost of which are sufficient moisture and the presence of a suitable food source. They are most abundant in soil and freshwater habitats.

In soils, Menapace et al. (1975) found that the abundance of Acanthamoeba and Hartmannella, as determined by the overlay-plaque technique, was as high as $3.2x10^3$ amoebae per gram of soil. Of the freshwater species studied by Page (1967), A. polyphaga was the most frequently isolated. Wright, Redhead & Maudsley (1981) identified acanthamoebae in 30 samples taken from sites which included fallow and cultivated soils, lake mud, river sediments and canal, lake and pond waters. Brown, Cursons & Keys (1982) isolated A. castellanii and A. polyphaga from Antarctic soils. Singh & Das (1972) identified A. culbertsoni and A. rhysodes from sewage sludge samples, whilst Nerad, Daggett & Sawyer (1982) isolated three species from polluted marine sediments. Acanthamoeba has also been reported from oceanic surface waters (Davis, Caron & Sieburth, 1978). Several of the species described by Pussard & Pons (1977) were isolated from swimming pools. Other sources of Acanthamoeba have included the internal tissues of the arenicolous basidiomycete Laccaria trullisata (Napolitano & Flanagan, 1980), air conditioning filters (Visvesvara et al., 1982) and bottled mineral waters (Rivera et al., 1981).

Jahnes, Fullmer & Li (1957) identified acanthamoebae as contaminants in monkey kidney tissue cultures. Acanthamoeba from a similar source was found to be pathogenic to mice and monkeys (Culbertson, Smith & Minner, 1958). More recently, acanthamoebae have been identified as potential pathogens of man, causing skin lesions (Gullet et al., 1979), meningoencephalitis (Willaert & Stevens, 1976)

and keratitis (Key, Willaert & Stevens, 1980).

The extensive distribution of these amoebae is undoubtedly a reflection of their ability to adapt readily to varying environmental conditions and utilize a range of different food sources.

Antagonists of cyanobacteria

It is clear from the preceding sections that protozoa and cyanobacteria often populate similar habitats, both aquatic and terrestrial. As a consequence many opportunities exist for interaction between the two groups. This thesis is concerned with some aspects of the predation of cyanobacteria by protozoa, specifically *Acanthamoeba* spp.. Before giving a detailed review of the present knowledge of this subject it is as well to place it in perspective by describing the other ways in which cyanobacterial populations may be antagonized. Such antagonists include macro-organisms (both invertebrate and vertebrate) as well as representatives of a number of microbial groups.

(A) Macrobial antagonists

(1) Invertebrates

As prominent components of the phytoplankton, cyanobacteria are potentially susceptible to grazing by zooplankters such as microcrustacea and rotifers. Although reports differ as to the extent and importance of cyanobacterial utilization by microcrustacea, it is clear that the ability and efficiency of grazing are largely determined by the size of food particle that the various crustacean groups can handle. Thus Porter (1973) found that the unicellular,

coccoid Chroococcus limneticus was effectively grazed and suppressed by Daphnia galeata and Diaptomus minutus, whereas the larger filamentous Anabaena spp. were too large to ingest and so unaffected by grazing. Johnson, Xu & Sieburth (1982) reported that although Synechococcus cells were ingested by the calanoid copepod Calanus finmarchicus, they persisted undigested and passed out intact in the faeces.

Microcrustacea can also be important predators in waterlogged and/or flooded soils, particularly paddy fields, where cyanobacteria often provide a source of low-cost nitrogen for rice production. In flooded soils, four out of five strains of nitrogen-fixing cyanobacteria were found to support reproducing populations of the ostracod Cypris sp. (Grant & Alexander, 1981). Each day an ostracod could consume a dry weight of Tolypothrix equivalent to its own dry weight. Recorded ostracod densities of 5 000-15 000/m^2 in parts of the Philippines suggest that grazing by these organisms may have a considerable influence on cyanobacterial populations (Grant & Alexander, 1981). Wilson, Greene & Alexander (1980) found that grazing by Cypris sp. prevented the development of inocula of Tolypothrix tenuis and suppressed inocula of Anabaena sp. added to flooded soil, whilst Osa-Afiana & Alexander (1981) found that increases in ostracod numbers and biomass were directly related to decrease in biomass of Aulosira and Anabaena.

Large-scale predation of cyanobacteria by insects has been reported from the alkaline thermal springs (40-55°C) of Yellowstone National Park, U.S.A. (Wickstrom & Wiegart, 1980). Brine flies are the main agents responsible for the breakdown of gelatinous mats of

cyanobacteria and bacteria once they become exposed to air and cool. Both adults and larvae of ephrydid fly species, most commonly Paracoenia turbida and Ephydra bruesii, invade, feed on and eventually solubilize the mat. The principal cyanobacteria consumed are Mastigocladus laminosus, Phormidium spp. and Synechococcus lividus (Wickstrom & Wiegart, 1980).

(2) Vertebrates

Many waters of tropical and warm temperate regions, in which cyanobacteria dominate the phytoplankton, contain herbivorous fish which utilize cyanobacterial primary production (Hickling, 1961). For example, in Lake George, Uganda, Haplochromis nigripinnis feeds mainly on Microcystis colonies, whilst the commercially exploited Tilapia nilotica utilizes most of the phytoplankton species, of which 70% are cyanobacteria, including Microcystis aeruginosa and Anabaenopsis spp. (Moriarty & Moriarty, 1973). Tilapia was found to assimilate a maximum of 70-80% of ingested carbon from Microcystis and Anabaena.

The endorheic alkaline saline lakes of East Africa develop vast populations of the planktonic filamentous cyanobacterium Spirulina platensis, and these, together with benthic diatoms, support a population of approximately 500 000 lesser flamingo, Phoeniconaias minor (Tuite, 1979). The flamingoes filter cyanobacteria from the lake using their modified bills. Populations of Spirulina in lake Texcoco in Mexico have also been exploited as a food source by birds. Gomara, secretary to Cortes, wrote that "...so many birds come to the lake for this food, that often in winter some parts are covered with them" (Farrar, 1966).

There are several records of cyanobacteria having been used as a food source by man. The vending of small cakes of cyanobacteria in the market of the capital city of Mexico in 1521 was described by Bernal Diaz del Castillo (Ciferri, 1981). The cakes, known as Tecuitlatl, were composed of dried mats of Spirulina maxima collected from the alkaline waters of L.Texcoco. In the Kanem region of the Republic of Chad, the present inhabitants consume dried biscuits (Dihé) composed entirely of Spirulina platensis (Ciferri, 1981). In both these cases the source of the cyanobacteria is highly alkaline water from which other algae are virtually excluded. The Spirulina thus grows as a quasi-monoculture which may be harvested with ease. More recently, cyanobacteria (especially Spirulina spp.) have attracted attention as a potential source of single cell protein (SCP) for human and livestock consumption.

(B) Microbial antagonists

Micro-organisms known to be antagonistic to cyanobacterial populations include bacteria, viruses, fungi and protozoa. For reviews of this subject see Stewart & Daft (1976, 1977) and Wright (1986).

For present purposes, antagonism may be defined as any action by an organism towards a cyanobacterium which leads to the inhibition of growth or loss of the cellular integrity of the cyanobacterium. In most cases the antagonism results in cell lysis, but sub-lethal effects are also possible, although less easy to detect. The various interactions differ markedly in their degree of specificity and recognition. The antagonism may be fortuitous (as is often the case with secondary metabolites), or it may be a highly specific

interaction (as with cyanophages). The mechanisms employed in antagonism include phagocytosis, entrapment and lysis, antibiosis, extracellular enzyme production and internal parasitism (see Fig.1.1). Different modes of attack may exist within a single group of antagonists, (e.g. fungi cause lysis by intracellular parasitism or by antibiotic production) and different groups of antagonist may employ similar methods (both bacteria and fungi exhibit antibiosis towards cyanobacteria). The following sections are arranged from a taxonomic viewpoint rather than on a mechanistic basis. Predation of cyanobacteria by protozoa is reviewed in the next section (p21).

(1) Viruses

Viruses attacking cyanobacteria were first reported from waste-stabilization ponds in the U.S.A. by Safferman & Morris (1963), who isolated and partially purified a virus (LPP-1) affecting the genera Lyngbya, Plectonema and Phormidium. Since then many examples of cyanobacterial viruses (cyanophages) have been reported from a wide range of geographical locations including Russia (Rubenchik et al., 1966) and India (Singh & Singh, 1967). Cyanophages readily produce plaques on lawn cultures of susceptible cyanobacteria. Unicellular, colonial, and both heterocystous and non-heterocystous filamentous cyanobacteria are all liable to cyanophage infection. Cyanophages have a rapid rate of multiplication. LPP-1 DUN has a generation time of 10h and a burst size of 100 particles per cell (Daft, Burnham & Yamamoto, 1985). Viral infection may affect the host cells in various ways prior to lysis, for example by inhibiting or destroying the ability of the host to photosynthesize.

Their position as obligate parasites dictates that the range of habitats occupied by cyanophages closely parallels those of their hosts, and includes rivers, lakes, ponds, rice paddy soils and sewage works (Stewart & Daft, 1977).

(2) Bacteria

Bacteria antagonistic to cyanobacteria are widely distributed in freshwater and soil habitats. They usually have a broad host range and as facultative pathogens can be cultured in isolation from the host. Groups of bacteria which lyse cyanobacteria include Cellvibrio spp., actinomycetes, myxobacteria, Bdellovibrio and bacilli.

(a) Cellvibrio

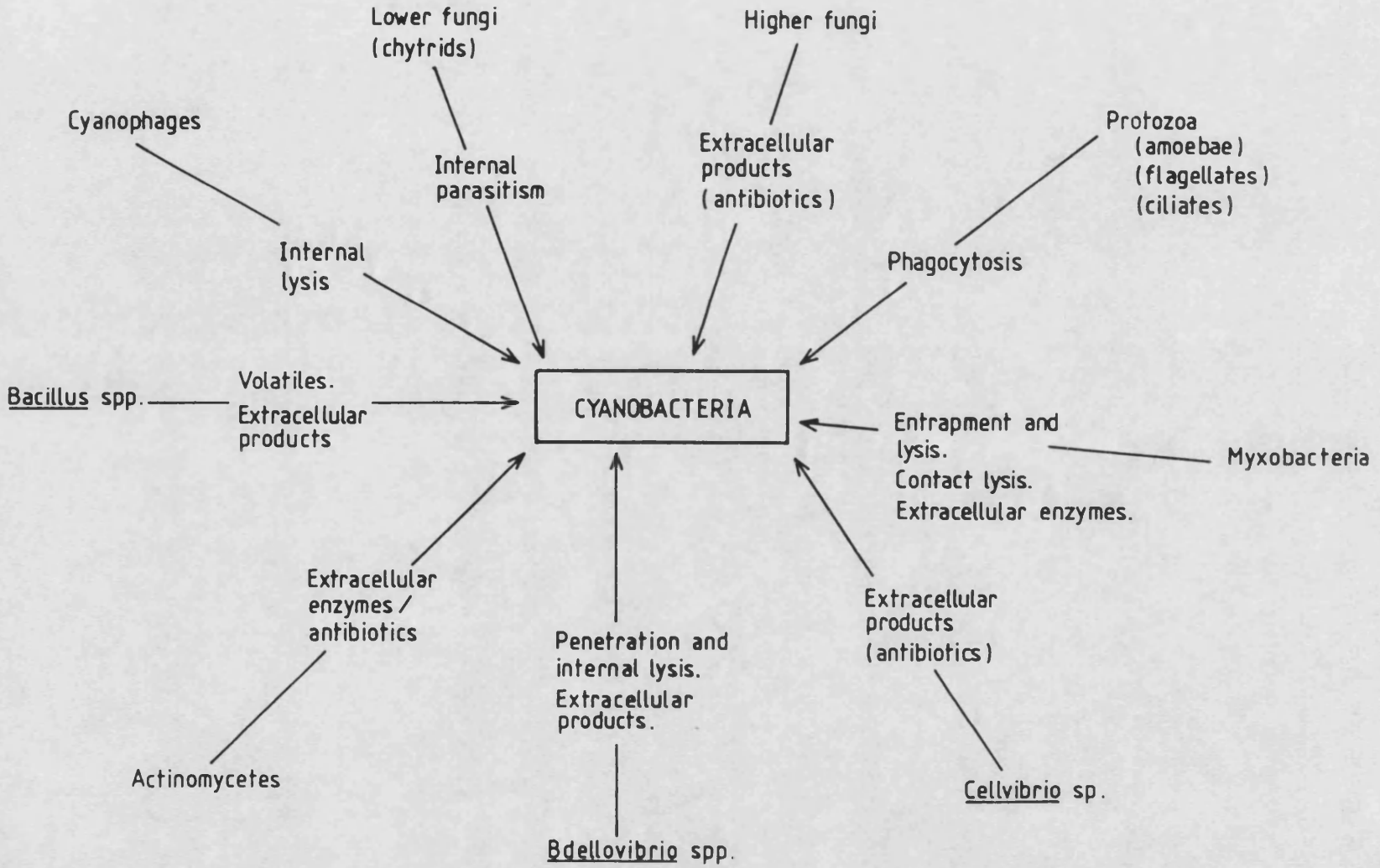
Granhall & Berg (1972) studied two strains of Cellvibrio which produced an antibiotic effective against six species of cyanobacteria. The active compound was soluble, heat-resistant and of low molecular weight, and was only produced in stationary phase cultures. Effects on the cyanobacteria included trichome breakage, sphaeroplast formation and cell lysis. These are similar to the effects of penicillin on cyanobacteria in that the mode of action prevents cell wall synthesis. The inhibition of cyanobacteria was reversible in most cases.

(b) Actinomycetes

Of 403 actinomycetes isolated from soil, Safferman & Morris (1962) found 24.6% to produce extracellular compounds effective against cyanobacteria, Plectonema boryanum being especially susceptible. Rubenchik, Bershova & Knizhnik (1965) reported 28 strains

Fig.1.1. Types and modes of action of microbial antagonists of cyanobacteria.

(Adapted from Daft, Burnham & Yamamoto (1985) and Wright (1986)).



of Streptomyces that inhibited growth of Anabaena hassallii, and Whyte, Maule & Cullimore (1985) reported the extracellular lysis (possibly by lysozyme) of Anabaena cylindrica by Streptomyces achromogenes. Redhead & Wright (1978) isolated a number of cyanobacteria-lysing Streptomyces spp.. The active compounds were soluble, diffusible and heat-stable (Wright, 1986). Sharma et al. (1984) found that extracellular products (possibly antibiotic) of a Streptomyces sp. caused lysis of Anabaena cylindrica.

(c) Fruiting myxobacteria

Stewart & Brown (1970) reported on four species of fruiting myxobacteria (Myxococcus xanthus, M. fulvus, Myxococcus sp. and Sorangium sp.) that lysed cells of Nostoc muscorum. Wu, Hamdy & Howe (1968) found seven cyanobacteria to be lysed by an organism tentatively identified as a Myxococcus sp.

Burnham, Collart & Highison (1981) have described the entrapment and lysis of Phormidium luridum by Myxococcus xanthus isolated from farm drainage ditches. In liquid culture Myxococcus formed colonial sphaerules up to 6mm in diameter within which Phormidium cells were degraded, probably by extracellular enzymes. Cyanobacteria were continually ensnared and transported to the centre of the colony for digestion. The colony interior provided a protected environment where digestive enzymes could be concentrated, increasing efficiency by avoiding dilution to the surrounding medium. By similar logic nutrients liberated from the prey organism were also concentrated, so facilitating absorbtion by myxococcal cells.

(d) Non-fruiting myxobacteria

Gromov et al. (1972) found Flexibacter flexilis var. algavorum (isolated from a naturally-occurring Nostoc population) to be antagonistic towards species of Anabaena, Nostoc and Phormidium. The lytic action depended on contact and occurred 3-4 days after inoculation onto cyanobacterial lawn cultures, and after 6-7 days in liquid culture. The anti-cyanobacterial activity of Flexibacter flexilis has also been reported by Redhead & Wright (1978), who isolated strains effective against several cyanobacteria, particularly Anabaena flos-aquae and A. cylindrica.

The most extensively studied non-fruiting myxobacteria antagonistic to cyanobacteria are strains FP-1 (Shilo 1970), CP-1 (Daft & Stewart 1971; 1973) and Myxobacter 44 (Stewart & Brown 1969; 1971). All these are Gram-negative rods and produce clearance plaques on cyanobacterial lawns in 1-7 days. CP-1 is a member of the genus Lysobacter (Christensen & Cook, 1978). The mode of action of Myxobacter 44 is by production of extracellular enzymes resembling lysozyme, whilst CP-1 and FP-1 require contact with the host for lysis to occur. In the initial stages of attack, CP-1 and FP-1 attach themselves end-on to the cyanobacterial cells, frequently close to the cross-septa in filamentous species. Penetration is initiated by lysis of the peptidoglycan layer in the cell wall. Dissolution of the remaining layers results in the release of the cell protoplasm into the surrounding medium. It is probable that several enzyme systems are involved in cell lysis. Under optimal conditions lysis occurs within 30 minutes of attachment of the bacterium to the host cell wall. Heterocysts and akinetes are more resistant to attack than vegetative

cells.

(e) Bdellovibrio

Wilkinson (1979) reported the presence of Bdellovibrio-like bacteria as endoparasites of cyanobacterial symbionts of coral reef sponges, where they caused distortion and disintegration of thylakoid membranes. Caioli & Pellegrini (1984) found that Bdellovibrio-like bacteria caused lysis of cyanobacterial cells in a natural bloom of Microcystis aeruginosa. Lysis only occurred after penetration of Microcystis by the bacteria, which located themselves mainly within the cyanobacterial periplasmic space. The Bdellovibrio reproduced by multiple fission within the periplasmic space of the host cells. Lysis began with the breakdown of cell organelles and led eventually to severe disruption of the host cell wall.

Non-penetrative lysis of cyanobacteria by Bdellovibrio may also occur. Burnham, Stetak & Locher (1976) described the gradual lysis of Phormidium luridum var. olivacea, Oscillatoria sp. and Microcystis aeruginosa by a heat-stable extracellular factor produced by Bdellovibrio bacteriovorus. Cell-free supernatants of Bdellovibrio cultures added to Phormidium caused more than 70% inhibition of photosynthesis in less than 24h, with lysis of Phormidium cells occurring in three to seven days. Susceptibility of the cyanobacteria to lysis was 2 to 3-fold greater in the dark compared with illuminated conditions.

(f) Bacilli

Wright & Thompson (1985) have reported that Bacillus spp. liberate volatile(s) which inhibit the growth and cause lysis of cyanobacteria, particularly Anabaena spp. In addition, Wright (unpublished) has observed cyanobacterial inhibition and lysis by aqueous diffusible product(s) of Bacillus spp.. Reim, Shane & Cannon (1974) isolated a strain of Bacillus brevis from a sewage oxidation pond which had a lytic effect on seven species of cyanobacteria. Lysis was extracellular, the active agent (probably antibiotic) being heat stable and of low molecular weight. Bacillus culture filtrates caused lysis of all cyanobacteria within 72h.

(3) Fungi

The fungal antagonists of cyanobacteria so far identified are mainly members of the chytridiales, the most extensive studies having been undertaken by Canter (see Canter, 1972, and references therein). These fungal pathogens are difficult to culture, so most information has been derived from observations of natural populations. Nevertheless, they are of worldwide distribution (Stewart & Daft, 1976) and probably represent important components of freshwater ecosystems. The chytrid genera most commonly involved are Rhizosiphon, Rhizophydium, Chytridium, and Phylctidium. Host cyanobacteria include species of Anabaena, Aphanizomenon, Gomphosphaeria, Lyngbya, Microcystis and Oscillatoria (Canter, 1972). The mode of antagonism of chytrid fungi is by physical penetration of the cyanobacterial cells. Depending on the species, between one and 20 cyanobacterial cells may be destroyed by infection from a single chytrid zoospore. In one form

of attack a fungal zoospore forms a sporangium in the mucilage surrounding the cyanobacterial filament from which a rhizoidal system develops. This may either grow along the outside of the filament, producing structures that penetrate individual cells (as is the case with Scherffeliomyces sp. parasitic upon Anabaena) (Canter, 1972) or alternatively grows through the cells comprising the filament (as with Rhizophydium megarrhizum parasitic upon Oscillatoria). The life cycle is completed by the formation and subsequent release of zoospores. In a second form of attack (e.g. Rhizosiphon crassum parasitic upon Anabaena) (Canter, 1951), the chytrid zoospore encysts within the host sheath before producing a fine thread which penetrates a cyanobacterial cell. A protosporangium develops from which a rhizoidal system grows through adjacent vegetative cells. The fungal protoplasm eventually emerges from the trichome to form a sporangium from which zoospores are released.

The distribution of chytrids usually parallels the abundance of the host species, although the latter may be destroyed if conditions are particularly favourable for fungal growth. Paterson (1960) found the parasitism of Anabaena planktonicum by Rhizosiphon anabaenae to be greatest at high oxygen concentrations, falling dramatically when oxygen saturation fell below 80%.

Besides chytrids, two other lower fungi antagonistic towards cyanobacteria have been described. Canter & Willoughby (1964) reported a parasitic species of Blastocladiella, B.anabaenae, which infected Aphanizomenon flos-aquae as well as species of Anabaena, and Canter (1973) has reported an unidentified species of biflagellate parasitic upon Anabaena solitaria. Infection of a bloom of Anabaena circinalis

by B. anabaenae has been described by Reynolds (1975).

Higher fungi may also be antagonistic towards cyanobacteria. Redhead & Wright (1978) isolated five species of cyanobacterial-lysing filamentous fungi from soil and aquatic habitats ; Acremonium charticola, A. kiliense, Emericellopsis minima, E.salmosynnemata and Verticillium lamellicola. These all caused lysis of Anabaena flos-aquae, and in most instances a number of other filamentous and unicellular cyanobacteria, in both lawn and liquid culture. Acremonium and Emericellopsis also inhibited the growth of cyanobacteria. In these two genera lysis was associated with diffusible, heat-stable extracellular factors. In further studies (Redhead & Wright, 1980) cephalosporin C was extracted and partially purified from both Acremonium and Emericellopsis. This, together with the high similarity between the effects of pure cephalosporin C and exudates of A. kiliense on Anabaena flos-aquae cells, provided strong evidence that the mode of antagonism is by cephalosporin production. At the cell level, the antibiotic probably serves by disrupting cell wall (specifically peptidoglycan) synthesis.

Daft (1981, cited in Daft, Burnham & Yamamoto, 1985) has reported a species of Cephalosporium which lysed ten out of twelve cyanobacteria, the lytic activity being associated with extracellular products.

Protozoan predation of cyanobacteria

In contrast to the groups of antagonist described in the foregoing section (particularly viruses, bacteria and fungi) the protozoa have been largely ignored as potential predators of cyanobacteria. Studies such as those of Canter & Lund (1968) and Canter (1973) have demonstrated the impact that protozoan predation can have on eukaryotic algae, especially Chlorophyta and Bacillariophyta. However, the types of detailed study performed with other groups of microbial antagonist have yet to be applied to protozoa. The reasons for this are partly historical, microbiologists having tended in the past to exclude protozoa from the scope of their investigations, but in addition there are more practical explanations. Amongst these are the difficulties inherent in the accurate identification of protozoa (especially small free-living amoebae), and also in their successful isolation and culture.

Despite these problems, there are a number of publications citing instances of predation of cyanobacteria by protozoa. These include both field and laboratory investigations and range from incidental observations to detailed ultrastructural and cytochemical studies. They are here subdivided into those referring to flagellates, amoebae and ciliates. A complete listing of these protozoa by taxonomic group, together with the cyanobacterial species consumed, is presented in Table 1.2. Large scale predation of massed cyanobacteria ("blooms") in natural situations is considered in more detail later.

(A) Flagellates

Reports of predation of cyanobacteria by flagellates relate almost exclusively to ingestion by members of the nutritionally versatile Order Chrysomonadida, and in particular to species of Ochromonas.

Daley, Morris & Brown (1973) described the predation of Anacystis nidulans by an Ochromonas sp. isolated as a contaminant in open cultures of the cyanobacterium. Addition of small amounts of contaminated culture to axenic Anacystis cells led to an increase in numbers of Ochromonas and complete destruction of the Anacystis population within 6 to 24h, depending on the inoculum size. Disappearance of Anacystis resulted from phagotrophic ingestion by Ochromonas, and not from the effects of associated bacteria or of soluble products released by the flagellate. Transmission electron microscopy of mixed cultures revealed numerous cells of Anacystis at different stages of digestion within Ochromonas food vacuoles. The recognized ochromonad, O. danica, also predated Anacystis nidulans.

Prows & McIlhenny (1973) reported the predation of Microcystis aeruginosa by four species of Ochromonas : O. bastrop, O. ovalis, O. malhamensis and O. danica. The latter association (that of O. danica and M. aeruginosa) was also studied by Cole & Wynne (1974, 1975), who used light and electron microscopy as well as cytochemistry to examine the endocytosis and digestion of cyanobacterial cells. Accumulation of Microcystis within the Ochromonas caused the flagellates to adopt a spherical profile instead of their usual pyriform shape.

Ochromonas granularis has been reported to ingest "oscillaria"

(Sandon, 1932) and the same author lists an additional three Chrysomonad species which utilize cyanobacteria.

Of the other flagellate groups, the dinoflagellate Ceratium hirundinella has been observed ingesting filaments of Anabaena (Sandon, 1932). The only zooflagellate reported to consume cyanobacteria is the choanoflagellate Stephanoeca diplocostata (Laval, 1971).

(B) Amoebae

Amongst the early reports of cyanobacteria being subject to attack by amoebae, Leidy (1879) referred to predation of "blue-green algae" by Amoeba radiosa, Amoeba verrucosa and Amoeba proteus. The latter species, usually regarded as a carnivore, has also been reported as a predator of Oscillatoria (Picken, 1937). Ho & Alexander (1974) found that cultures of Amoeba discoides and Amoeba radiosa were predatory upon several cyanobacteria including Anacystis nidulans and three species of Anabaena. Tetramitus rostratus ingested three of seven cyanobacteria tested. Disruption of phagocytosed cyanobacteria by these amoebae was attributed to the production of lysozyme-like enzymes. Comandon & De Fonbrune (1936) described the predation of Oscillatoria spp. by Amoeba verrucosa and another, unidentified amoeba for which the Oscillatoria constituted the major food source. Haberey (1973a, 1973b) presented light and electron microscope studies of the phagocytosis of Oscillatoria by Thecamoeba sphaeronucleolus. Other lobose amoebae which consume cyanobacteria are the omnivorous Pelomyxa palustris (Lee, Hutner & Bovee, 1985), and a Pelomyxa sp. reported by

Reynolds (1971) as predatory upon a bloom of Aphanizomenon. Whitton (1973) observed that under natural conditions small amoebae frequently occurred within mucilaginous masses of cyanobacteria and that these, at least in the case of Microcystis, predated the cyanobacterial cells.

Huang & Wu (1982) isolated two predatory amoebae, an Amoeba sp. and a Nuclearia sp., from rice paddy fields by their ability to form plaques on Anabaena lawns. Both species could be maintained with Anabaena as the sole prey, and in addition consumed species of Nostoc, Oscillatoria and Tolypothrix. Nuclearia ingested all types of Anabaena cell, but only vegetative cells were digested, akinetes and heterocysts being voided intact from the cell. In static liquid culture both Amoeba and Nuclearia severely limited Anabaena populations, the decline in prey resulting in encystment of amoebae. Predation of cyanobacteria by Nuclearia has also been reported by Yamamoto (1981) who isolated a strain from the surface waters of L.Kasumigaura, Japan, which formed plaques on lawns of Microcystis aeruginosa, Anacystis nidulans and Anabaena cylindrica. On the basis of frequency of plaque formation amoebae were the commonest microbial agents causing lysis of cyanobacteria in the lake, occurring more frequently than either bacteria or fungi. It was suggested that such amoebae may be active in the regulation of cyanobacterial blooms in the lake waters. Laboratory investigations (Yamamoto, 1981) yielded similar results to those of Huang & Wu (1982), with cyanobacterial decline stimulating encystment of amoebae. Further studies with this Nuclearia have been reported by Yamamoto & Suzuki (1984) and Daft, Burnham & Yamamoto (1985). The former authors, in a comparison of the

Table 1.2. Literature reports of protozoan predation of cyanobacteria

Taxon[1]	Predator	Prey	Reference(s)
PHYLUM SARCOMASTIGOPHORA			
Subphylum Mastigophora			
Class Phytomastigophorea			
Order Chrysomonadida	Brehmiella chrysohydra	"Blue-green algae"	(36)
	Chrysarachnion insidians	"Minute cyanophyceae"	(36)
	Chrysodendron ramosum	Blue-green algae"	(36)
	Ochromonas sp.	Anacystis nidulans	(12)
	O.bastrop	Microcystis aeruginosa	(32)
	O.danica	"	(6,7,32)
	O.granularis	"Oscillaria"	(36)
	O.malhamensis	Microcystis aeruginosa	(32)
	O.ovalis	"	(32)
Order Dinoflagellida	Ceratium hirundinella	Anabaena	(36)
Class Zoomastigophorea			
Order Choanoflagellida	Stephanoeca diplocostata	"Cyanobacteria"	(23)
Subphylum Sarcodina			
Class Lobosea			
Order Amoebida	Acanthamoeba sp.	Anabaena spp.	(15)
		Nostoc spp.	(15)
		Oscillatoria sp.	(24)
	A.castellanii	Anabaena cylindrica	(19)
		A.flos-aquae	(11,19,43,44)
		A.inequalis	(19)
		Anacystis nidulans	(43,44)
		Gloeocapsa alpicola	(43,44)

		Phormidium luridum	(11)
		Plectonema boryanum	(44)
	Amoeba sp.	Anabaena sp.	(20)
		Nostoc sp.	(20)
	A.discoides / A.radiosa	Anabaena cylindrica	(19)
		A.flos-aquae	(19)
		A.inequalis	(19)
		Anacystis nidulans	(19)
		Nostoc sp.	(19)
	A.radiosa	Gloeotrichia echinulata	(19)
	A.proteus	"Cyanobacteria"	(26,30)
	A.verrucosa	Oscillatoria sp.	(8)
	"Amoebae"	Microcystis sp.	(42)
	Thecamoeba sphaeronucleolus	Oscillatoria sp.	(17,18)
Order Schizopyrenida	Tetramitus rostratus	Anabaena cylindrica	(19)
		A.inequalis	(19)
		Gloeotrichia echinulata	(19)
Order Pelobiontida	Pelomyxa sp.	Aphanizomenon flos-aquae	(34)
	P.palustris	"Blue-green algae"	(25)
Class Eumycetozoea			
Order Physarida	Chondrioderma sp.	Nostoc	(36)
Class Filosea			
Order Aconchulinida	Asterocaelum anabaenophilum	Anabaena planctonica	(9,10)
	Nuclearia sp.	Anabaena sp.	(20)
		A.cylindrica	(45,46)
		A.flos-aquae	(11)
		Anacystis nidulans	(45,46)
		Microcystis aeruginosa	(45,46)
		M.elabens	(46)
		M.flos-aquae	(46)
		Nostoc sp.	(20)
		Oscillatoria sp.	(20)
		Phormidium luridum	(11,46)

		Tolypothrix sp.	(20)
	N.delicatula	Phormidium foveolarum	(4)
	Nucleosphaerium tuckeri	Phormidium foveolarum	(5)
		P.uncinatum	(5)
	Vampyrella sp.	Anabaena flos-aquae	(33)
Class Granuloreticulosea			
Order Athalamida	Arachnula impatiens	Anabaena cylindrica	(27)
		Aphanocapsa sp.	(27)
		Chlorogloea fritschii	(27)
		Nostoc muscorum	(27)
		Oscillatoria spp.	(13)
Class Heliozoea			
Order Actinophryida	Actinophrys oculata	"Oscillaria"	(36)
PHYLUM CILIOPHORA			
Class Kinetofragminophorea			
Order Prostomatida	Holophrya gracilis	"Blue-green algae"	(36)
	H.hexatricha	"	(36)
	H.perlucida	"	(36)
	Mesodinium pulex	"	(36)
	Trachelius trachelioides	Oscillatoria limosa	(36)
		O.rubescens	(36)
Order Trichostomatida	Sonderia macrochilus	"cyanophyceae"	(36)
	S.vorax	Oscillatoria	(36)
Order Colpodida	Bryophrya spp.	Nostoc	(36)
	Colpoda patella	"	(36)
	C.reniformis	"	(36)
	C.steinii	Anacystis nidulans	(1,2)
	Woodruffia spp.	Oscillatoria	(36)
Order Nassulida	Furgasonia rubens	"Cyanophyceae"	(36)
	Leptopharynx eurystoma	"Nostocaceae"	(36)
	Nassula aurea	Anabaena lutea	(31)

	A.torulosa	(31)
	A.variabilis	(31)
	A.viguieri	(31)
	"Cyanophyceae"	(36)
	Cylindrospermum alatospermum	(31)
	C.marchicum	(31)
	Gloeocapsa sp.	(31)
	Lyngbya aeruginosa	(31)
	Mastigocladus anabaenoides	(31)
	Nostoc pruniforme	(31)
	Oscillatoria sp.	(39)
	O.acutissima	(31)
	O.agardhii	(14)
	O.brevis	(31)
	O.formosa	(31)
	O.limosa	(31)
	O.ornata	(31)
	O.tenuis	(31)
	Phormidium autumnale	(31)
	P.inundatum	(31,40)
	P.retzii	(31)
	P.uncinatum	(31)
	P.unicinatum	(40)
N.citrea	Oscillatoria sp.	(41)
N.ornata	Anabaena flos-aquae	(3)
	"Cyanophyceae"	(36)
	Oscillatoria agardhii	(3)
	O.brevis	(3)
	O.limosa	(31,3)
	O.tenuis	(31)
	Phormidium sp.	(3)
N.picta	Oscillatoria formosa	(31)
	Phormidium autumnale	(31)
	P.inundatum	(31)
N.timida	Oscillatoria	(36)
Pseudomicrothorax agilis	"Nostocaceae"	(36)
P.dubius	Anabaena ambigua	(29)

		A.inequalis	(29)
		Oscillatoria animalis	(29)
		O.formosa	(28,29)
		O.lutea	(29)
		Phormidium autumnale	(28,29)
Order Cyrtophorida	Dysteria armata	"Cyanophyceae"	(36)
	Chilodonella sp.	Oscillatoria sp.	(30)
	Chlamydodon pedarius	"	(22)
Class Oligohymenophorea			
Order Hymenostomatida	Frontonia algivora	Oscillatoria	(36)
	F.depressa	Nostoc sp.	(36)
	F.elliptica	"Blue-green algae"	(36)
	F.leucas	Lyngbya sp.	(37)
		Oscillatoria sp.	(16,37)
		O.prolifera	(16)
	Ophryoglena atra	Anabaena circinalis	(35)
Order Scuticociliatida	Uronema sp.	Synechococcus sp.	(21)
Class Polyhymenophorea			
Order Heterotrichida	Condylostoma vorticella	Gomphosphaeria sp.	(38)
		Microcystis aeruginosa	(38)
		Synechococcus sp.	(38)

[1](The taxonomic system followed is that of Levine et al., 1980).

NOTE: Key To References

(1) Bader et al. (1976a)
(2) Bader et al. (1976b)
(3) Brabrand et al. (1983)
(4) Cann (1986)
(5) Cann and Page (1979)
(6) Cole and Wynne (1974)
(24) Lazcano et al. (1977)
(25) Lee et al. (1985)
(26) Leidy (1879)
(27) Old and Darbyshire (1978)
(28) Peck (1977)
(29) Peck (1985)

(7) Cole and Wynne (1975)
(8) Comandon and De Fonbrune (1936)
(9) Cook and Ahearn (1976)
(10) Cook et al. (1974)
(11) Daft et al. (1985)
(12) Daley et al. (1973)
(13) Dobell (1913)
(14) Eberly (1959)
(15) Ghabbour et al. (1980)
(16) Goldsmith (1922)
(17) Haberey (1973a)
(18) Haberey (1973b)
(19) Ho and Alexander (1974)
(20) Huang and Wu (1982)
(21) Johnson et al. (1982)
(22) Kaneda (1960)
(30) Picken (1937)
(31) Poilvert (1959)
(32) Prows and McIlhenny (1973)
(33) Prowse (1968)
(34) Reynolds (1971)
(35) Reynolds (1975)
(36) Sandon (1932)
(37) Schaeffer (1920)
(38) Takamura and Yasuno (1983)
(39) Thomas (1941)
(40) Tucker (1978)
(41) Webb (1956)
(42) Whitton (1973)
(43) Wright et al. (1979)
(44) Wright et al. (1981)
(45) Yamamoto (1981)

suitability of various microbial food sources, found that cyanobacteria were by far the most readily consumed, green algae being ingested to a much lesser extent and bacteria not at all. Of six cyanobacterial species tested, Phormidium luridum was the most extensively predated. Anabaena cylindrica was ingested, although heterocysts and akinetes remained intact (Yamamoto & Suzuki, 1984). Cann (1986) has maintained Nuclearia delicatula with filaments of Phormidium foveolarum as the food source. Sections of trichome 50µm in length were routinely ingested.

Closely related to Nuclearia are two other filose amoebae, the Vampyrella sp. reported by Prowse (1968) to attack a bloom of Anabaena flos-aquae, and Asterocaelum anabaenophilum which predates bloom-forming populations of Anabaena planctonica (Cook et al., 1974; Cook & Ahearn, 1976). Nucleosphaerium tuckeri, originally isolated from pond water in Scotland, is predatory upon species of Phormidium (Cann & Page, 1979).

Dobell (1913) described the predation of cyanobacteria, especially small "oscillariae", by Arachnula impatiens, and Old & Darbyshire (1978) recorded the ingestion of four genera of cyanobacteria by the same amoeba, which in addition consumed bacteria, flagellates, diatoms and nematodes.

Several authors have reported predation of cyanobacteria by Acanthamoeba spp.. Lazcano, Benitez & De La Nieto (1977) found Acanthamoeba as a contaminant predating cultures of Oscillatoria, and Ghabbour et al. (1980) implicated a species of Acanthamoeba in the destruction of Anabaena and Nostoc in desert soils, although direct evidence of this was not obtained. Ho & Alexander (1974) found that

Acanthamoeba castellanii was predatory upon four species of cyanobacteria in lawn culture, with Anacystis nidulans and Nostoc sp. resisting ingestion. Seven species of eukaryotic algae, including three species of Chlamydomonas, were also consumed. Wright et al. (1979) and Wright, Redhead & Maudsley (1981) have described the predation of unicellular and filamentous cyanobacteria in lawn and liquid culture by several Acanthamoeba spp., particularly Acanthamoeba castellanii. Of the cyanobacterial species tested, Anabaena flos-aquae and Gloeocapsa alpicola were particularly susceptible to predation, whilst others including Anabaena cylindrica and Nostoc muscorum were rejected. Eukaryotic algae (Chlamydomonas spp., Chlorella vulgaris, and Coelastrum microporum) were ingested to a limited extent by some strains. Exhaustion of cyanobacteria led to encystment of amoebae, whilst renewal of the food source resulted in excystment and renewed predation.

There is only one report of cyanobacterial predation by heliozoa, that of "oscillaria" by Actinophrys oculata (Sandon, 1932). The same author records ingestion of Nostoc by the myxamoeba Chondrioderma.

(C) Ciliates

(1) Kinetofragminophorea

"Blue-green algae" were recorded as a major food source for the kinetofragminophorans Mesodinium pulex and three species of Holophrya (Sandon, 1932). The same author listed predation by both Trachelius trachelioides and Sonderia vorax upon species of Oscillatoria, and of S. macrochilus upon "cyanophyceae". Amongst the Colpodida, species of

Bryophrya fed exclusively on Nostoc and Woodruffia spp. predated Oscillatoria (Sandon, 1932). Colpoda spp. have been reported to consume both Nostoc (Sandon, 1932) and Anacystis (Bader, Tsuchiya & Fredrickson, 1976a, 1976b). The latter authors investigated predation of Anacystis nidulans by Colpoda steinii in both batch and chemostat culture. In dark-incubated batch cultures a marked reduction in cyanobacterial population due to predation by ciliates was followed by encystment of Colpoda. With illuminated chemostat cultures a steady state of coexistence was reached in which populations of both predator and prey remained stable. Anacystis was only slightly less suitable than Escherichia coli as a food source for Colpoda when measured in terms of the specific reproduction rate of the protozoan.

Filamentous cyanobacteria, especially species of Phormidium and Oscillatoria, constitute the sole food source for many ciliates of the order Nassulida, including species of Nassula and Pseudomicrothorax. Poilvert (1959) made a comprehensive survey of the food range of three species of Nassula. With N. aurea, nine out of the twenty cyanobacteria tested supported abundant growth of the ciliate, the remainder allowing only limited proliferation. N. picta preyed on Phormidium autumnale, P. inundatum and Oscillatoria formosa, and could be maintained indefinitely with these species as food. N. ornata only ingested Oscillatoria limosa and O. tenuis, with the latter being considered the usual food. The prey range of N. ornata has also been investigated by Brabrand et al. (1983), who recorded proliferation of the ciliate when fed three species of Oscillatoria, a Phormidium species and a strain of Anabaena flos-aquae. Two other Anabaena strains, a toxic strain of A. flos-aquae and A. solitaria, were

rejected, as was Microcystis aeruginosa.

Thomas (1941) found that blooming of Oscillatoria in samples of lake water left to stand in the laboratory was followed by the development of high densities of N. aurea, the predatory activity of which caused considerable reduction of the cyanobacterial population. Eberly (1959) described the predation of a bloom of Oscillatoria agardhii by a planktonic population of Nassula aurea, and Webb (1956) reported the ingestion of Oscillatoria in brackish waters by N. citrea. The distribution of N. citrea was strictly determined by the presence of Oscillatoria, the ciliate only occurring in association with abundant filaments of the cyanobacterium. In freshly collected samples the Nassula were always coloured green by the contents of food vacuoles, and ceased feeding once the supply of Oscillatoria had been exhausted despite the availability of diatoms and other algae. Many of the starved ciliates encysted (Webb, 1956).

Pseudomicrothorax dubius feeds on species of Phormidium, Oscillatoria and Anabaena (Peck, 1977; 1985), and Sandon (1932) recorded ingestion of Nostocaceae by Pseudomicrothorax agilis and Leptopharynx (Trichopelma) eurystoma.

Picken (1937) reported a species of Chilodonella (Chilodon) to be predatory upon mats of Oscillatoria. Ordinarily the Oscillatoria was ignored as a food source, instead providing a substratum over which the ciliates moved in search of diatom prey. However, in ageing mats, on which diatom numbers were much reduced, the Oscillatoria was actively attacked by the Chilodonella. Trichomes several times the length of the ciliate were ingested and coiled within the cell.

Kaneda (1960) maintained the cyrtophorine ciliate Chlamydodon

pedarius in culture with Oscillatoria as food.

(2) Oligohymenophorea

Sandon (1932) recorded the predation of cyanobacteria by three species of Frontonia, and Schaeffer (1920) found Oscillatoria and Lyngbya to be important components of the diet of Frontonia leucas. Trichomes of Oscillatoria six to eight times the length of the Frontonia were readily taken in through the cytostome by coiling of the filament within the ciliate. A marine scuticociliate, Uronema sp., has been maintained in culture for over a year with an oceanic isolate of Synechococcus (and associated bacteria) as food (Johnson, Xu & Sieburth, 1982). During exponential growth the Uronema preferentially selected the Synechococcus, cyanobacterial numbers declining by three orders of magnitude compared with a decrease of one order of magnitude in the bacterial population. Reynolds (1975) reported the predation of a natural bloom of Anabaena circinalis by Ophryoglena atra.

(3) Polyhymenophorea

The only report of ingestion of cyanobacteria by polyhymenophorans is that of Takamura & Yasuno (1983) for Condylostoma vorticella in L.Kasumigaura, Japan. Condylostoma ingested planktonic populations of both prokaryotic and eukaryotic algae. Of the cyanobacteria, the ciliates fed selectively on Microcystis, Synechococcus and Gomphosphaeria, whilst largely rejecting species of Phormidium and Aphanizomenon. The size and shape of the cyanobacteria were the main determinants of their suitability as prey.

(4) General comments

A number of comments should be borne in mind when considering these reports. For example, some of the interactions between predator and prey are inadequately described, particularly in the older literature. Thus Sandon (1932) frequently referred to protozoa as feeding on "blue-green algae" or "nostocaceae" without stating the precise species consumed. Description of species in insufficient detail, together with the attachment of different names to a single species by separate authors, has sometimes made it difficult for a species described in the literature to be assigned with confidence to a currently recognized genus. The situation is compounded by some authors themselves being uncertain of the identity of isolates, particularly in the case of small freshwater and soil amoebae.

As with all laboratory-based observation, the dangers of extrapolating results to field situations cannot be over-emphasized. Laboratory experiments show what food is acceptable to a protozoan, but do not necessarily reflect the natural food choice (Nisbet, 1984). For many of the reports based on laboratory studies there is no evidence that the protozoan ingests the stated cyanobacterial prey in natural circumstances, although if both predator and prey populate the same habitat it is possible, even probable, that predation occurs. Even then, alternative food sources may be utilized preferentially by virtue of their size, abundance or superior nutritive value. In other cases predator and prey may be ecologically isolated and so prevented from coming into contact.

Only positive interactions are usually recorded; reports indicate which cyanobacteria are ingested but rarely state what was

unacceptable, since few reports are of ordered feeding trials from which such information can be gathered. Nor do many reports state whether the protozoan is capable of proliferating on a diet consisting solely of the prey species, or whether additional food sources are required. Such observations, when available, provide useful information on the nutritional value of particular prey species. Studies in which predator and/or prey are in non-axenic culture have added complications in that it is difficult to assess the relative contributions of the cyanobacterium and associated bacteria to the nutrition of the protozoan.

As seen from Table 1.3, the majority of protozoa reported as predatory upon cyanobacteria are ciliates (31 species), both amoebae and flagellates having approximately half this number of predatory species. Few conclusions can be drawn from the relative representation of the three protozoan groups, which more probably reflects their ease of culture and popularity as research organisms than their ecological importance as cyanobacterial predators. Likewise, cyanobacteria used to "screen" cultures of protozoa for their predatory ability are invariably selected on the basis of what is available to the researcher rather than selecting those species which the protozoan is most likely to encounter in nature.

Despite these drawbacks, the published reports, taken as a whole, provide a broad overview of the types of interaction which occur. Doubtless many more examples could be discovered by further laboratory experimentation and by observation of natural protozoan communities.

Table 1.3. The number of genera and species of protozoa reported as predatory upon cyanobacteria.

Group	Genera	Species
Flagellates	7	12
Amoebae	12	15
Ciliates		
Kinetofragminophorea	14	24
Oligohymenophorea	3	6
Polyhymenophorea	1	1
Total	37	58

(5) Large-scale predation of cyanobacterial blooms

Reliable information on the extent and effectiveness of predation in natural habitats is best gained by direct observation. However, references in the literature to naturally-occurring instances of predation of cyanobacteria by protozoa (defined as the observation of freshly collected samples as opposed to laboratory-based feeding trials or selective isolation and culture from field samples) are scarce. Foremost of the reasons for this lack of data is that direct observation and identification of microbial predators is only possible in situations where high densities of the cyanobacterium, and consequently the predator, are able to develop over a considerable area; yet under most circumstances microbial populations in the environment are too low for direct observation to be practicable. Thus in soil habitats the overall amount of predation may be considerable, although at any one time the actual numbers of predator and prey will be too small for direct observation. In addition, conditions suitable for proliferation of cyanobacteria and their subsequent predation by protozoa may fluctuate dramatically both spatially and temporally within the component microhabitats of a soil, further reducing the probability of detection and resulting in a false impression of the extent of predation.

In contrast to soils, very high concentrations of cyanobacteria can develop in aquatic habitats given that certain physical, chemical, and biological conditions are satisfied. Unlike soils, aquatic habitats are often characterized by considerable homogeneity over a large area, so permitting the synchronous development of cyanobacterial populations. In these circumstances the accompanying

increase in protozoan predators, and their effect on the cyanobacteria, may be sufficiently obtrusive to attract comment. For these reasons, the following reports of large-scale predation of cyanobacteria relate without exception to antagonism of cyanobacterial "blooms". The reports are mostly incidental to the main aim of the research, which may imply that other instances of predation have been observed but not reported. With amoebae, the scarcity of information may reflect the rapidity of their appearance and disappearance and the fragility of the trophic stage (Cook & Ahearn, 1976).

Cook et al. (1974) reported that blooming of Anabaena planctonica in a freshwater lake in Georgia, U.S.A. was followed by a large increase in the population of a Mayorella-like amoeba. Surface masses of cyanobacteria were reduced to a milk-like slick 1-2 mm thick over a period of 1-3 days. The amoebae, up to 300 µm in diameter, were actively and exclusively predating the Anabaena cells and so responsible for the decline in cyanobacterial population. Trichomes of more than 20 cells were ingested and disrupted within two minutes, although heterocysts could not be digested. The amoeba was subsequently described as a new species of Asterocaelum, A. anabaenophilum (Cook & Ahearn, 1976), closely related to Asterocaelum algophilum which feeds exclusively on centric diatoms (Canter, 1973). Asterocaelum anabaenophilum produced large, spiny cysts which remained in the surface scum of the lake (Cook & Ahearn, 1976). The amoebal-cyanobacterial association was very common, recurring over a number of years, and was considered the primary cause of disappearance of the annual cyanobacterial bloom. The amoebal population appeared to be dependent on the growth of Anabaena planctonica. Asterocaelum was

not detectable in the Anabaena population prior to surface massing of the cyanobacteria, which was the trigger for amoebal proliferation.

Prowse (1968) noted the attack on cells of an Anabaena flos-aquae bloom in a fish culture pond by an amoeba, tentatively identified as a Vampyrella sp.. As with Asterocaelum, the amoeba was considered to be the major cause of breakdown of the bloom.

Eberly (1959) found that the decline in a bloom of Oscillatoria in Myers Lake, Indiana, U.S.A., was partly correlated with the grazing pressure of an immense population of the planktonic ciliate Nassula aurea, which fed exclusively on the cyanobacterium. Grazing was concentrated on the lower surface of the Oscillatoria bloom where temperatures (3-6°C) were most favourable to the Nassula, the Oscillatoria being restricted to a thin stratum as a consequence. Nassula densities as high as 12 816/1 were recorded, with values in excess of 1 000/1 not uncommon.

Amoebal predation of a bloom of Aphanizomenon flos-aquae in Crose Mere, Shropshire, England was reported by Reynolds (1971). In the summer of 1966 the Aphanizomenon population reached a maximum of 5 900 filaments/ml on 4 July, the end of this maximum coinciding with "the presence of a rapidly multiplying rhizopod (tentatively identified as Pelomyxa sp.), individuals of which were seen to be packed with blue-green coloured vacuoles and recognizable Aphanizomenon heterocysts ; numbers were drastically reduced to less than one filament/ml by mid-July". Predation of an Anabaena circinalis bloom, this time by a ciliate, was reported from the same body of water in 1973 by the same author (Reynolds, 1975). The maximal population of A. circinalis occurred on 22 May (37 000 cells/ml),

although by that time it was subject to intense grazing by a large population of the ciliate Ophryoglena atra (maximum density 120/l). In addition, many cells were infected by the chytrid Blastocladiella anabaenae. As a consequence the Anabaena population rapidly collapsed to 560 cells/ml by 29 May.

Feeding mechanisms in the predation of cyanobacteria by protozoa

Protozoa have evolved diverse and sometimes very complex mechanisms for obtaining food. Most free-living species take particulate matter into the cell by phagocytosis, and this is how protozoa feed on cyanobacteria. In many amoebae phagocytosis simply involves the invagination and pinching-off of part of the plasma membrane to form a food vacuole enclosing the food particle(s). The undifferentiated nature of many species means that phagocytosis can occur over much of the cell surface. By contrast, ciliates have a highly differentiated cortex made up of the epiplasm, alveoli, kinetosomes and fibrillar systems. This layer must be circumvented to allow direct contact between the plasmalemma and endoplasm during food intake. Consequently, phagocytosis in ciliates is usually restricted to a structurally elaborate oral region. Many species have cytopharyngeal specializations below the cytostome (mouth) adapted to deal with particular types of prey. In flagellates phagocytosis normally occurs at the anterior end, close to the base of the flagella.

It seems that few protozoa preferentially select cyanobacterial prey. Many species have a varied diet in which cyanobacteria are ingested along with algae, bacteria, yeasts and inanimate organic

particles. This is particularly the case with lobose amoebae, the undifferentiated nature of which enables acceptance of a wide range of prey sizes. Ciliates may feed on cyanobacteria by active filtration, creating a water current with their cilia from which food particles are extracted and packaged into food vacuoles at the cytostome. In these suspension-feeding species food selection is on the basis of particle size rather than the type of prey organism. Other ciliates may browse surfaces colonized by cyanobacteria.

Although predator and prey normally meet by chance contact, other factors may operate to make this more likely. For example, Webb (1956) found that Nassula citrea and its Oscillatoria prey were both positively phototactic, so providing a mechanism by which the ciliate could accumulate in areas where food was abundant. There is also evidence that Oscillatoria formosa produces a diffusible factor partly responsible for the accumulation of Pseudomicrothorax near cyanobacterial food sources (Peck, 1985).

Some details of the mechanisms by which protozoa phagocytically ingest cyanobacteria have been described in the foregoing sections. The accounts of predation given by different authors for different protozoan species contain many similarities. This is particularly so for filamentous cyanobacteria, in which contact between predator and prey is normally followed by attack at the end of the trichome. Side attacks may also occur, and in these the cyanobacterium is usually bent on itself and ingested as a double strand. Both ciliates and amoebae may ingest trichomes several times their own length, often coiling the cyanobacterium which leads to considerable distortion of the predator (Cook & Ahearn, 1976; Goldsmith, 1922; Picken, 1937;

Webb, 1956; Wright, Redhead & Maudsley, 1981). In Acanthamoeba a cylindrical pseudopodium is extended along and around the filament and convenient lengths (up to 15 cells) broken off for intracellular digestion (Wright et al., 1981). In the ciliate Frontonia leucas the cytostome is pushed over the end of the filament, ingestion being brought about by a combination of ciliary action, body movement and rotational streaming of cytoplasm (Schaeffer, 1920; Goldsmith, 1922). The mechanism by which the flagellate Ochromonas feeds on the unicellular Microcystis has been described by Cole & Wynne (1974). Cyanobacterial cells were enclosed in a primary endosome at the anterior of the Ochromonas , and this migrated posteriorly to fuse with the secondary endosome, into which it released its contents. Repetition of this process resulted in up to eight Microcystis cells accumulating in the secondary endosome, at which point further phagocytosis was suspended to allow digestion.

Peck (1985) has studied pre-ingestion feeding behaviour in Pseudomicrothorax dubius, dividing it into two phases, contact swimming and phagocytosis, the latter subdivided into attachment and ingestion stages. During contact swimming the ciliate moved along the Oscillatoria, the ventral cilia in contact with the filament. In side attacks the attachment step lasted 5-20 seconds, during which localized lysis of the filament was observed in the region of the cytostome. The filament was then folded and ingested. Various modifications in ciliary behaviour were observed during feeding. For example, the oral membranelles ceased beating during the attachment and ingestion stages.

The most specialized devices for the ingestion of cyanobacteria

are found in nassulid and cyrtophorid ciliates, which possess an organelle known as the cytopharyngeal basket. The morphology and functioning of these structures has been studied by several authors including Hausmann & Peck (1978; 1979), Peck & Hausmann (1980) and Tucker (1968; 1978). The principal components are large bundles of microtubules, the nemadesmata, arranged as a tube. In Pseudomicrothorax dubius the basket is 50μm in length (compared with a cell length of 60-80μm) and has on average 22 nemadesmata, each composed of approximately 200 cross-linked microtubules. Extending from each nemadesm towards the lumen of the basket is a lamella of 20-30 microtubules, each with two projecting arms. The wall of the basket is completely closed by a filamentous sheath extending between adjacent nemadesmata, with the exception of a perforated zone just below the region of attachment of the basket to the cell cortex. During phagocytosis filamentous cyanobacteria are engulfed at up to 15μm s^{-1} and are enclosed in food vacuole membrane immediately they enter the cytopharynx. This rapid vacuole growth is achieved by fusion of large numbers of membrane-bound vesicles which enter the lumen of the basket in the anterior perforated zone. Peck & Hausmann (1980) found these vesicles positive for acid phosphatase, identifying them as lysosomes. In this way the developing food vacuole receives a constant supply of digestive enzymes. These enzymes were capable of rapid penetration of the cyanobacterial cell wall, entering the cytoplasm in less than one second and destroying cellular integrity within five seconds. The lysosomes may also contain rapid-acting killing agents which increase the permeability of the cyanobacterial wall, so facilitating the entry of digestive enzymes. In addition the

host hydrolases may activate an autodigestive mechanism in the cyanobacterium (Peck & Hausmann, 1980).

Hausmann & Peck (1979) have proposed a model for force generation in which the arm-bearing microtubules of the nemadesmal lamellae propel the food vacuole (via filamentous material) into the cell by a cyclic pattern of attachment and reattachment analogous to the sliding filament hypothesis of ciliary motion. The structure and function of the cytopharyngeal basket of Nassula is essentially similar to that of Pseudomicrothorax dubius, the major difference being in the proposed mechanism of force transfer during the inward movement of filaments (Tucker, 1968; 1978).

Whatever the precise mechanisms of prey capture adopted by different protozoa, the cyanobacteria, once ingested, are enclosed in food vacuoles where they undergo digestion. Lysosomes fuse with the food vacuoles, releasing lytic enzymes which break down the cyanobacterial cells. Indigestible remains are eventually ejected from the cell. For an account of digestive processes in protozoa see Nisbet (1984). Presumably all protozoa which predate cyanobacteria share the ability to produce lysosome-like enzymes needed for the breakdown of the peptidoglycan layer of the cyanobacterial cell wall.

The food of Acanthamoeba

Although Acanthamoeba is usually regarded as a bactivore (Nisbet, 1984; Lee, Hutner & Bovee, 1985), reports in the literature indicate that species and strains of Acanthamoeba can obtain nutriment from a wide variety of sources besides bacteria. These include fungi, cyanobacteria (see p31), algae, vertebrate tissues and liquid media.

Castellani (1930a) originally isolated Acanthamoeba castellanii as a contaminant in cultures of the yeast Cryptococcus pararoseus. The amoeba was predatory upon the yeast cells, which were clearly visible within the amoebal protoplasm. In feeding trials with bacteria as the food source, Castellani (1930b) found that this amoeba formed plaques on lawn cultures of "Bacillus" typhosus, "B". paratyphosus A,B and C, "B". dysenteriae, "B". asiaticus and Monilia macedoniensis, whilst no clearing occurred with "Bacillus" morgani, these observations being confirmed by Douglas (1930). Van Rooyen (1932) studied the effects of the same strain of A. castellanii on bacterial cultures, and concluded that plaque formation was due to the mechanical ingestion of bacterial cells by the amoebae as opposed to the production of diffusible bacteriolytic agents or changes in the pH of the medium. Agar slope and plate cultures of living and dead Bacillus typhosus were completely cleared within seven days of inoculation with amoebae. The susceptibility to predation of the 91 bacterial strains tested showed no relationship either to Gram-reaction or to particular taxonomic groupings. Resistant strains became susceptible to predation following heat-killing and washing. A. castellanii could also be maintained in pure culture with dead cells of the yeast Cryptococcus pararoseus as the food source.

Nero, Tarver & Hedrick (1964) found that A. castellanii grew well on the yeast Torulopsis famata, each trophozoite ingesting 70 yeast cells a day when incubated at 18°C. A decrease in the yeast population was associated with an increase in amoebal numbers, the latter encysting as yeast cells became scarce. This A. castellanii isolate originated from soil, in conjunction with Candida parapsilosis which it also predated. Rapid growth was also obtained with a Bacillus sp. as food.

Neff (1957) noted that an Acanthamoeba sp. was unable to consume living cells of Saccharomyces cerevisiae, S. ellipsoideus or Schizosaccharomyces sp., but would grow on the autoclaved cells. Escherichia spp. and Aerobacter spp. were consumed both when living and when heat-killed.

Bryant et al. (1982) investigated the predation of Pseudomonas paucimobilis by Acanthamoeba polyphaga in sterilized soil samples which were kept either continually moist or subjected to wet-dry cycles. In moist soil amoebal grazing reduced bacterial populations to 20-25% of the level in ungrazed control populations, but was not so effective in the wet-dry treatments.

Ray (1951) reported five species of bacteria (including Pseudomonas fluorescens and Serratia marcescens) which could support growth of an Acanthamoeba sp.. Adam (1964) and Bovee (1963) found that strains of A. castellanii grew well on cultures of Aerobacter aerogenes, and Stratford & Griffiths (1978) used Klebsiella aerogenes to culture the same Acanthamoeba species.

Heal & Felton (1969), in studies on the relationship between amoebae and the microflora of soil, screened an Acanthamoeba sp. for

the ability to predate and to proliferate on a wide range of micro-organisms. Of 37 isolates of aerobic soil bacteria tested, all supported amoebal reproduction, with 43% supporting abundant reproduction. Comparison of a wide range of bacterial types showed that Gram-negative rods gave the most growth of amoebae. Predation was enhanced with Bacillus spp. and Chromobacterium spp. if the chains of cells were first broken into shorter lengths. Serratia marcescens would only support reproduction at 35°C, at which temperature no pigment (prodigiosin) was produced. Less than half of the actinomycetes tested (strains of Nocardia, Mycobacterium and Streptomyces) were capable of supporting amoebal reproduction. Saccharomyces and Kloeckera were the most susceptible of the 19 yeasts predated. Nine of the 14 soil algae were ingested, but only three supported reproduction of amoebae. No proliferation of amoebae occurred with sterilized organic matter as a food source.

A number of workers have succeeded in culturing Acanthamoeba spp. axenically in liquid media. The media used include 4% w/v mycological peptone (Adam, 1959), proteose peptone, glucose and yeast extract (Chagla & Griffiths, 1974) and various defined media (e.g. Adam, 1959).

Certain species and strains of Acanthamoeba are capable of proliferation on living vertebrate tissues, both in vivo and in vitro (see p 7).

Feeding mechanisms in Acanthamoeba

Endocytosis (phagocytosis and pinocytosis) is the sole means by which Acanthamoeba can obtain nutrients. Phagocytosis and pinocytosis are essentially two manifestations of the same process. Phagocytosis is the mechanism by which particulate matter enters the cell, whereas pinocytosis involves the non-selective uptake of solute molecules. Pinocytosis operates continuously in Acanthamoeba, and provides the means by which it can be cultured axenically in liquid media devoid of particulate matter (Bowers & Olszewski, 1972).

With A. castellanii, Bowers (1977) found that all endocytosis ceased below 5°C, and that it was reversibly stopped by inhibitors of aerobic metabolism such as sodium azide and cyanide. The combined volume of the two forms of uptake remained more or less constant, with an increase in phagocytosis leading to a suppression of pinocytosis and vice versa. On the basis of these observations it has been proposed that phagocytosis and pinocytosis have a common control mechanism, and that the volume of the cell internal compartment occupied by endocytosed material is critical in determining the maximum rate of endocytic uptake. The volume of this compartment, estimated by measuring the volume of latex beads that saturate the phagocytic mechanism, was approximately 15% of the amoebal cell volume (Bowers, 1977).

The supply of surface membrane is critical in endocytosis, since both phagocytosis and pinocytosis involve the invagination and pinching-off of plasma membrane. Bowers & Olszewski (1972) found that although the total volume of medium ingested in pinocytosis was relatively small (21% mean cell volume per hour), the surface to

volume ratio of pinocytic vesicles was large, leading to a high rate of membrane internalization. Membrane transfer was estimated to be 2-10 times the surface area of the amoeba each hour, much of this through recycling of existing membrane rather than de novo synthesis.

Phagocytosis in Acanthamoeba is thought to be a two-step process. Food particles are first bound to the surface of the amoebal cell (see below), and only subsequently engulfed. However, binding is not necessarily followed by phagocytosis (Bowers, 1977), this being dependent on a specific interaction at the cell surface which results in a localised stimulus for the cell to engulf the particle (Bowers & Olszewski, 1983). Uptake can be very rapid: Bowers (1980) found that bound particles could be surrounded by pseudopodia and internalized within 40s. There was no preferential uptake of particles at any particular region of the cell surface. This observation is in contrast to those of some workers, for example Page (1967), who described Acanthamoeba as feeding primarily by food cup formation on one side of the posterior end of the cell.

Ray (1951) has described a distinctive method of feeding in A. astronyxis by which the amoeba accumulates a large number of bacteria at the cell surface by agglutination. The bacteria attach with their flagella following chance contact with the amoeba. Whilst initially occurring over the entire cell surface, the bacteria subsequently shift to the posterior of the amoeba where they continue to accumulate. Phagocytosis of bacteria occurs in the proximal region of the agglutinated cells. A similar process has been observed for A. castellanii feeding on species of Pseudomonas, Proteus and Rhodospirillum by Preston, Davies & King (1981) and Preston & King

(1982), who have identified surface binding sites on the amoeba specific for flagellin. Brown, Bass & Coombs (1975) found that A. castellanii preferentially ingested horse erythrocytes over those from other sources due to binding of the cells to carbohydrate sensitive sites on the surface of the amoeba. By testing a range of potential food organisms a correlation was found between the ability of the prey to agglutinate to the amoebal cells and its ability to support amoebal growth.

Aims

The aims of the present study were to investigate various aspects of the predation of cyanobacteria by Acanthamoeba spp. These included determination of the types of cyanobacteria consumed, the capacity of Acanthamoeba to deplete cyanobacterial populations, and the extent to which cyanobacteria supported proliferation of amoebae. It was also hoped to obtain information about the way in which cyanobacterial prey are digested once they have been internalized by phagocytosis. Information from such studies should hopefully give a much clearer indication of the effectiveness of acanthamoebae as predators of cyanobacterial populations.

GENERAL MATERIALS AND METHODS

General

In all experimental procedures good microbiological practice was strictly observed. Particular care was taken in handling *Acanthamoeba* in view of the potential pathogenicity of some strains. Contaminated glassware was always completely immersed in 1% (v/v) Stericol solution (Sterling Industrial, Sheffield), and disposable materials were decontaminated by autoclaving.

Cultivation of amoebae

The stock cultures of *Acanthamoeba* maintained during this study are listed in Table 2.1. All *Acanthamoeba* strains were grown axenically in proteose peptone-glucose-yeast extract (PGY) medium (Chagla & Griffiths, 1974) consisting of, in g/l distilled water, proteose peptone 7.5, yeast extract 7.5, glucose 15.0. The pH was adjusted to 6.8 with dilute (0.1M) HCl and NaOH, and the medium autoclaved at 121°C for 15 min at 15lb/in^2.

Stock cultures (four replicates for each strain) were maintained in Universal bottles containing 10ml PGY medium inoculated with 0.5ml from an established culture. Amoebae were incubated statically in the dark at 27°C, and subcultured monthly.

Batch cultures were grown in 250ml Erlenmeyer flasks containing 50ml PGY medium inoculated with 2.5ml from an established culture (3d). Flasks were incubated at 30°C and agitated on a rotary shaker (80 oscillations min^{-1}). Subcultures were made every three days. Cultures were screened regularly for contamination by means of light microscopic observation and by streaking samples onto nutrient agar

plates (1.5%, w/v) followed by incubation at 30°C for 7d and inspection for the presence of bacterial and/or fungal colonies. Any contaminated cultures were immediately discarded.

Cultivation of cyanobacteria

The stock cultures of cyanobacteria maintained during this study are listed in Table 2.2. All cyanobacterial strains, with the exception of Spirulina spp., were grown in Allen's medium (Allen, 1968; see Appendix A for composition) prepared from 100-fold concentrated stock solutions of each constituent. The pH of the medium was adjusted to 7.8 with dilute (0.1M) HCl and NaOH. Spirulina was grown in a specialized medium of high bicarbonate concentration and high alkalinity (pH 11) (see Appendix A for composition).

Stock cultures of cyanobacteria were maintained in test tubes on agar (1.5%, w/v) slopes of the same media. Slopes were incubated at room temperature in a North-facing window, and subcultured at six-weekly intervals. Batch cultures were grown in 100ml volumes of the appropriate medium in 250ml Erlenmeyer flasks inoculated with 2ml from an established culture (21d). Flasks were incubated at 30°C under constant illumination from 40 Watt warm white fluorescent tubes (photosynthetically active radiation (PAR) at culture level of 17 microeinsteins m^{-2} sec^{-1}) and agitated on a rotary shaker at 80 oscillations min^{-1}. Subcultures were made every three weeks. All cyanobacterial cultures were periodically checked for bacterial and/or fungal contaminants in the manner previously described for amoebal cultures.

Table 2.1. Reference numbers and sources of Acanthamoeba stock cultures.

SPECIES	SOURCE*	STRAIN NO./CODE	ORIGIN
A.castellanii	1	CCAP 1501/1a	Soil, U.S.A.
"	1	CCAP 1501/2a	England
"	1	CCAP 1534/2	Freshwater, U.S.A.
"	1	CCAP 1534/3	Soil, England
"	2	NEFF G	-
"	3	BATH 31	-
"	3	BATH PB	Soil, England
A.polyphaga	1	CCAP 1501/3a	Freshwater,U.S.A.
"	1	CCAP 1501/3b	Freshwater,U.S.A.
A.palestinensis	1	CCAP 1547/1	Soil, Israel

* The sources of strains were as follows:

(1) Culture Collection of Algae and Protozoa, 36 Storey's Way, Cambridge, England.

(2) Dr.A.J.Griffiths, Department of Microbiology, University College, Cardiff, Wales.

(3) Isolated previously in this laboratory (Dr.S.J.L.Wright).

Table 2.2. Strain numbers and sources of cyanobacterial stock cultures.

SPECIES	SOURCE*	STRAIN NO./CODE	ORIGIN
Anabaena sp.+	2	DAFT A_4	-
Anabaena catenula	1	CCAP 1403/1	Freshwater, Holland
Anabaena cylindrica+	3	CARR	-
Anabaena cylindrica	1	CCAP 1403/2a	Freshwater, England
Anabaena variabilis+	3	CARR 7118 DM	-
Anacystis montana	1	CCAP 1430/0	Freshwater
Anacystis nidulans+	3	CARR	-
Aphanizomenon flos-aquae+	1	CCAP 1401/3	Freshwater, England
Calothrix parietina+	4	WHIT D550	-
Microcystis aeruginosa	1	CCAP 1450/1	Freshwater
Nostoc calcicola	1	CCAP 1453/1	Soil, Holland
Nostoc muscorum+	4	WHIT D584	-
Nostoc muscorum	1	CCAP 1453/12	Freshwater
Plectonema boryanum	2	DAFT 594	-
Spirulina sp.	5	WALS 13-TE	E.African soda lake
Spirulina sp.	5	WALS 24	E.African soda lake
Synechococcus leopoliensis+	1	CCAP 1405/1	Freshwater, U.S.A.
Tolypothrix tenuis	1	CCAP 1482/3a	Freshwater, Japan

(continued)

Table 2.2 (continued)

+ Axenic culture

* Sources of strains were as follows:

(1) Culture Collection of Algae and Protozoa, 36 Storey's Way, Cambridge, England.

(2) Dr.M.J.Daft, Department of Biological Sciences, The University, Dundee, Scotland.

(3) Dr.N.G.Carr, Department of Biochemistry, University of Liverpool, Liverpool, England.

(4) Dr.B.A.Whitton, Department of Botany, University of Durham, Durham, England.

(5) Prof.A.E.Walsby, Department of Botany, University of Bristol, Bristol, England.

Measurement of Acanthamoeba cell size

Trophozoites: A drop of amoebal suspension taken from a 2d batch culture was placed on a microscope slide and overlaid with a coverslip supported by paraffin wax along two sides. For each Acanthamoeba strain measurements were made, using a calibrated eyepiece graticule, of 50 trophozoites in their rounded-up form (diameter) and 50 trophozoites in the locomotory form (length and breadth).

Cysts: Cysts were produced by introducing trophozoites into cultures of Synechococcus leopoliensis 1405/1. Samples (2.5ml) from 3d batch cultures of Acanthamoeba were transferred to tubes and pelleted by centrifugation (500g for 10min). The supernatant was removed and the cells resuspended in the same volume (2.5ml) of sterile Allen's medium. Repli dish wells containing 2ml of a Synechococcus batch culture were inoculated with 130µl of the amoebal suspension and incubated at 26°C for 7d, by which time large numbers of cysts were present. Samples were examined microscopically, and measurements made of the diameter of 50 cysts of each strain.

Light microscopy/photography

A Leitz Dialux microscope (E.Leitz (Instruments) Ltd., Luton) was used for routine observations and cell counts, using either bright field or phase contrast optics. For photomicrography and differential interference contrast optics a Leitz Orthoplan microscope with Orthomat-W camera attachment was used. Kodachrome 64 slide film (64 A.S.A) (Kodak Limited, Hemel Hempstead, Herts.) was routinely employed

for colour photography, and Kodak Plus-X (125 A.S.A.) for monochrome.

For most microscopic observations material in liquid suspension was placed on a slide and overlaid with a coverslip, the latter supported with paraffin wax to reduce compression. Drying out of slide preparations was prevented by periodic irrigation with water. For microscopic observation of lawn cultures, material was scraped from the surface of the agar with a sterile wire loop, mixed with a drop of water on the slide, and overlaid with a coverslip.

Amoebal/cyanobacterial counts

Counts of amoebal and cyanobacterial populations were made using a Modified Fuchs-Rosenthal haemocytometer (supplied by Richardsons of Leicester Ltd., Leicester). In each case the number of cells in all nine squares of the haemocytometer grid were counted. The dimensions of the chamber were 0.2x3x3mm, giving a volume of $1.8mm^3$

From this the number of cells/ml could be calculated as:

$$\frac{\text{Count}}{1.8} \times 1000$$

The number of counts made on each sample varied according to the experiment. Precise details are given in the materials and methods sections relevant to each experiment. For filamentous cyanobacteria counts were made of the number of cells as opposed to the number of filaments present.

SECTION ONE

THE CYANOBACTERIAL PREY RANGE OF ACANTHAMOEBA

Introduction

This introductory section investigates the ability of Acanthamoeba spp. to predate a range of cyanobacteria in both lawn and liquid culture. Also included in this section are some observations on the characteristics of plaque formation by acanthamoebae on cyanobacterial lawns.

Materials and methods

Determination of the cyanobacterial prey range of Acanthamoeba spp.

(1) On lawn cultures

Lawn cultures of cyanobacteria were grown on Allen's medium agar (1.5% w/v; 25ml) in 9cm diameter plastic Petri dishes (Sterilin Ltd.). After setting, the agar surface was air-dried for 1h prior to inoculation with 2ml from an established cyanobacterial batch culture (approx 14d). The inoculum was evenly distributed over the surface of the agar by tilting the dish. Plates were left to stand on a flat surface for 15-25 min to allow settling of cyanobacterial cells onto the agar surface. Excess fluid was drained off by inversion of the dishes, which were then incubated (lid-uppermost) in constant light (photosynthetically active radiation (PAR) of 45 microeinsteins $m^{-2}s^{-1}$) at 30°C (normally) for 2d to allow development of a confluent cyanobacterial growth. Each lawn was then given three point inocula (10µl) of Acanthamoeba from a 2d batch culture, and a control (10µl)

of sterile PGY medium. Inoculated lawns were incubated as above.

(2) In liquid culture.

The range of cyanobacteria predated by Acanthamoeba in liquid culture was determined by incubating mixtures of the two organisms in Repli dish wells. For cyanobacteria, batch cultures (approx. 14d) were used as the inoculum. In cases where cyanobacterial growth was particularly dense, cultures were diluted with sterile Allen's medium before being dispensed. To prepare amoebal inocula, samples (5ml) from a 2d batch culture in PGY medium were centrifuged (10 min at 500g), the supernatant removed, and the cells resuspended in 3.5ml Allen's medium taken from 100ml of the same medium in a 250ml Erlenmeyer flask. This amoebal suspension was then diluted by transferal to the remaining Allen's medium in the flask.

Three experimental and two control wells were established for each Acanthamoeba / cyanobacterial combination. Experimental wells contained 1.5ml cyanobacterial suspension and 1.5ml of the diluted Acanthamoeba suspension. Control wells contained 1.5ml Allen's medium in place of the amoebal inoculum. Repli dishes were incubated at 20°C and were continuosly illuminated (PAR of 13 microeinsteins $m^{-2}s^{-1}$). Samples were removed at intervals for microscopic examination.

Scanning electron microscopy of cyanobacterial lawns

Lawns of Microcystis aeruginosa 1450/1 were prepared as described above. Lawns received a single, central inoculum (10µl) of A. castellanii G from a 2d batch culture and were incubated in the light at 30°C while plaques developed. Lawns with a regular, well

developed plaque (approx 1.5cm in diameter) and without any visible evidence of regrowth of cyanobacteria within the cleared area were selected for scanning electron microscopy (SEM).

Small agar blocks approximately 0.8 x 0.4cm in area were excised from the lawn surface. Each block was cut so that it spanned the edge of a plaque. All but the upper 2mm of each block was sliced away and discarded. The remaining portions were placed separately in small plastic cylinders, the ends of which were sealed with filter paper discs. The filter paper was perforated by numerous pin-sized holes, so allowing the passage of liquids into and out of the capsule whilst minimizing mechanical disturbance to the agar surface and attached microorganisms.

Specimens were fixed for 1h at 4°C in 2% (v/v) osmium tetroxide in 0.1M sodium cacodylate buffer, pH 6.8. After fixing the agar blocks were washed by resuspension in distilled water for 10min, this process being repeated three times. Preparations were then dehydrated by passage through a graded acetone series (30%, 50%, 75%, 75%, 90%, 90%, 100%, 100%, 100%), with 20min in each solution. Specimens were left overnight in 100% acetone to ensure the complete removal of water from the agar blocks. Fixed, dehydrated blocks were critical point dried, mounted on aluminium stubs and coated with gold in a Polaron 5000 sputter coater (Polaron Equipment Ltd., Watford, Herts.). Specimens were viewed using a Jeol 35C scanning electron microscope (Jeol U.K. Ltd., Colindale, London) operated at 25 KV.

Results

Predation of cyanobacterial lawn cultures

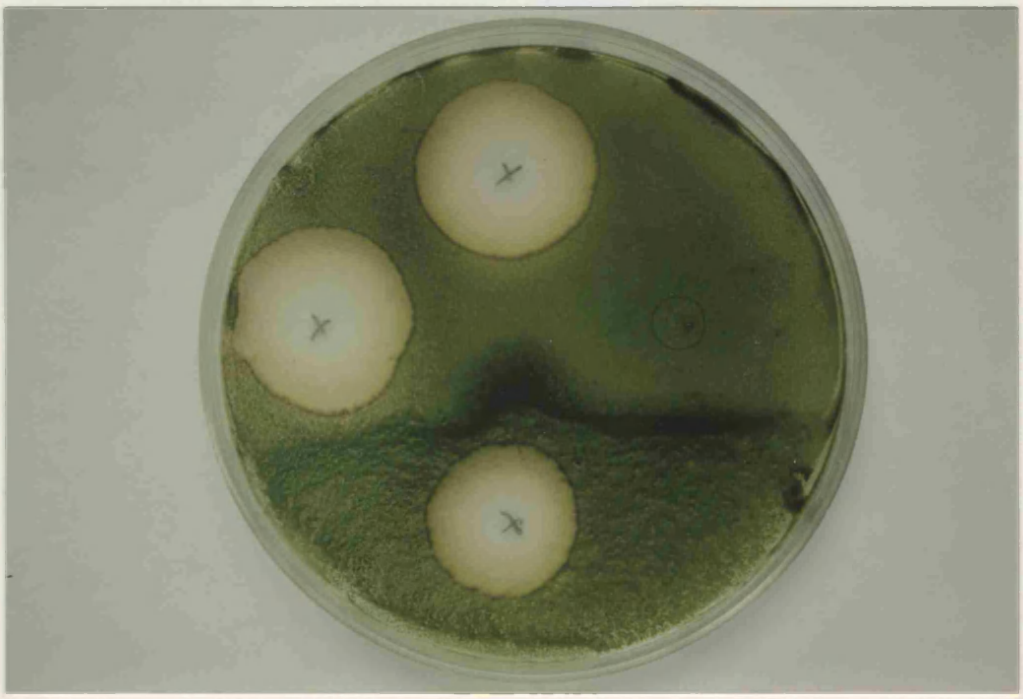
(1) General observations

Addition of point inocula (10µl) of *Acanthamoeba* spp. to confluent lawn growths of susceptible cyanobacteria (see below) led to the formation of cleared areas or plaques on the lawn surface. Plaque formation, when it occurred, was similar for all *Acanthamoeba* / cyanobacterial combinations. In consequence the following observations apply in general to all such interactions. Clearing was first evident (usually after 2d) as a slight patchiness within the area of lawn over which the inoculum had spread (approx. 0.5cm in diameter). Once this region of the lawn became completely clear of cyanobacteria, the area of the plaque increased concentrically from the point of inoculation. Fig.3.1 shows the progressive development of plaques on a single lawn culture of *Anacystis nidulans* CARR inoculated with trophozoites of *Acanthamoeba castellanii* PB. In many cases separate plaques on a lawn culture increased to the extent that they fused with one another. No plaques developed from controls (sterile PGY medium). Usually there was a clear demarcation between the edge of the plaque and the surrounding ungrazed cyanobacterial growth. The region of the lawn just within the plaque boundary often appeared slightly green, indicating the presence of some ungrazed cyanobacteria.

Microscopic observation of scrapings taken from the edge of plaques showed that clearance of the cyanobacterial growth was the direct result of ingestion (phagocytosis) of cyanobacterial cells by *Acanthamoeba* trophozoites. Many of the amoebae contained large numbers of cyanobacterial cells visible within food vacuoles. With filamentous

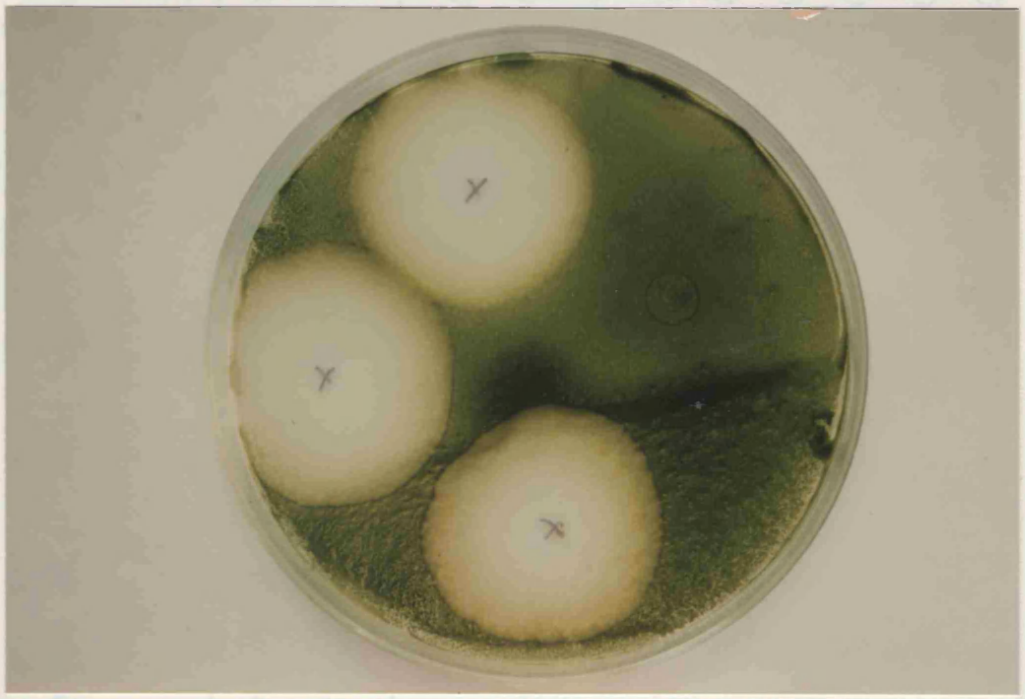
Fig.3.1. Predation of lawn cultures of Anacystis nidulans CARR by Acanthamoeba castellanii PB. Development of clearance plaques after 5,9 and 13 days incubation (all photographs are of the same lawn)*.

5 days incubation



9 days incubation

Fig.3.1. (Continued).



13 days incubation

*The lawn received three point inocula (10µl, positions marked by crosses) of amoebae from a 3d batch culture and a single control (arrowed) of sterile PGY medium (10µl). Incubation was at 20°C in the light.

Table 3.1 The dimensions of actively-moving trophozoites, rounded-up trophozoites and cysts of ten strains of *Acanthamoeba*[*]

Species	Strain No./code	Cyst diameter (μm)	Rounded trophozoites	Locomotory trophozoites	
			Diameter (μm)	Length (μm)	Breadth (μm)
A. castellanii	CCAP 1501/1a	17.6 (1.2)	25.8 (1.6)	39.2 (2.1)	22.4 (1.4)
"	CCAP 1501/2a	17.5 (0.7)	20.9 (0.9)	33.8 (2.0)	22.1 (1.5)
"	CCAP 1534/2	15.2 (0.7)	20.6 (0.8)	29.8 (1.7)	15.0 (0.8)
"	CCAP 1534/3	15.5 (0.7)	21.7 (0.8)	24.2 (1.3)	17.2 (0.8)
"	NEFF G	13.8 (0.7)	18.1 (0.8)	24.9 (1.2)	14.2 (0.7)
"	BATH 31	13.3 (0.9)	18.1 (0.7)	23.0 (1.2)	14.9 (1.0)
"	BATH PB	18.8 (1.0)	24.1 (0.9)	33.6 (1.8)	15.9 (0.8)
A. polyphaga	CCAP 1501/3a	17.3 (0.6)	20.3 (0.7)	33.4 (2.1)	23.5 (1.4)
"	CCAP 1501/3b	14.6 (0.7)	24.1 (1.4)	35.7 (2.3)	23.7 (1.6)
A. palestinensis	CCAP 1547/1	20.6 (0.9)	23.4 (0.9)	39.7 (2.5)	24.1 (2.1)

[*]All values are the mean of 50 measurements.
Figures in parenthesis are plus/minus 95% confidence intervals.

species (e.g. Plectonema boryanum, Anabaena spp.) trichomes of greater length than the amoebae were readily ingested (see Table 3.1 for the dimensions of amoebal strains used in this study). In such cases cyanobacteria were coiled within the amoebae, often causing considerable distortion of cell shape. Sometimes amoebae were observed to simultaneously ingest two cyanobacterial filaments, one at each end of the cell. When ingesting filamentous species, Acanthamoeba typically extended a tubular pseudopodium around and along the trichome.

In samples taken from the centre of established plaques (e.g. 7d after inoculation of amoebae) the cyanobacterial population was totally (or almost totally) exhausted by the grazing pressure of amoebae. However, very few trophozoites were present, the amoebal population being composed almost exclusively of cysts (often at very high density).

(2) Scanning electron microscopy of lawn cultures

Scanning electron microscopy (sem) was used to observe undisturbed lawn cultures of Microcystis aeruginosa 1450/1 inoculated with A. castellanii strain G. Fig.3.2 shows a sequence of electron micrographs of a single agar block (0.8x0.4cm) excised from the surface of a lawn. The sequence extends from the ungrazed cyanobacterial growth, across the plaque boundary, and on into the plaque proper. Ungrazed regions of the lawn consisted of an uninterrupted multilayer of Microcystis cells (Fig.3.2 (B)). The maximum penetration of Acanthamoeba trophozoites was only 1mm beyond the visible boundary of the plaque. The quality of specimen

Fig.3.2. Predation of lawn cultures of Microcystis aeruginosa 1450/1 by Acanthamoeba castellanii G.

Scanning electron micrographs of a single agar block (and attached micro-organisms) excised from a lawn culture of Microcystis (the section of lawn removed contained both part of a plaque and part of the ungrazed area).

(A) Low magnification micrograph of the agar block, the surface of which is divided into two areas, the ungrazed cyanobacterial lawn growth (L), and the cleared region of the plaque (P). The sharp separation of the two areas is clearly shown (dotted line). (Bar marker = 500μm).

(B) to (H). Sequence of micrographs of the agar surface from the region of ungrazed cyanobacterial growth, across the plaque boundary and into the plaque. (In all cases distances were measured each side of the plaque edge, and refer to the centre of each micrograph).

(B) 2.0mm into Microcystis lawn. The cyanobacterium forms an uninterrupted, multi-layered growth. No amoebae are present. (Bar marker = 100μm)

(C) 1.0mm into lawn. Only one Acanthamoeba cell is visible (arrowed). (Bar marker = 100μm)

(D) 0.5mm into lawn. Large numbers of trophozoites (arrowed) are mixed in with the cyanobacterial cells. The distribution of amoebae is weighted to the right of the micrograph (the side nearest the plaque edge). (Bar marker = 100μm)

(E) Plaque edge. The Microcystis population is much reduced compared with the ungrazed region of the lawn. (Bar marker = 100μm)

(F) 0.5mm into plaque. (Bar marker = 100μm)

(G) 1.0mm into plaque. Very few Microcystis cells remain (arrowed). A = exposed agar surface. (Bar marker = 100μm)

(H) 2.5mm into plaque. The agar surface is covered by an uninterrupted layer of Acanthamoeba cysts. (Bar marker = 100μm)

Fig. 3.2

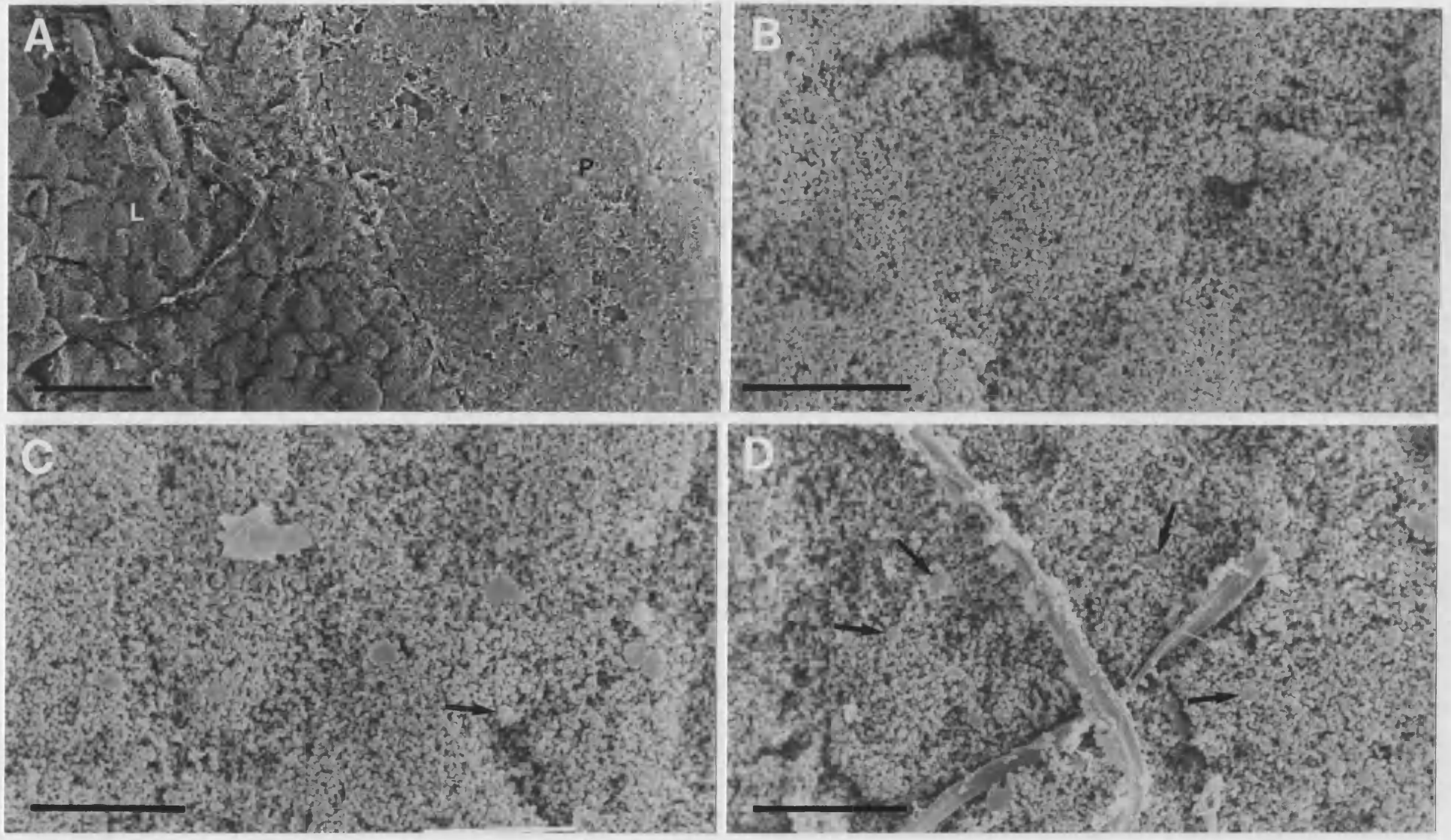
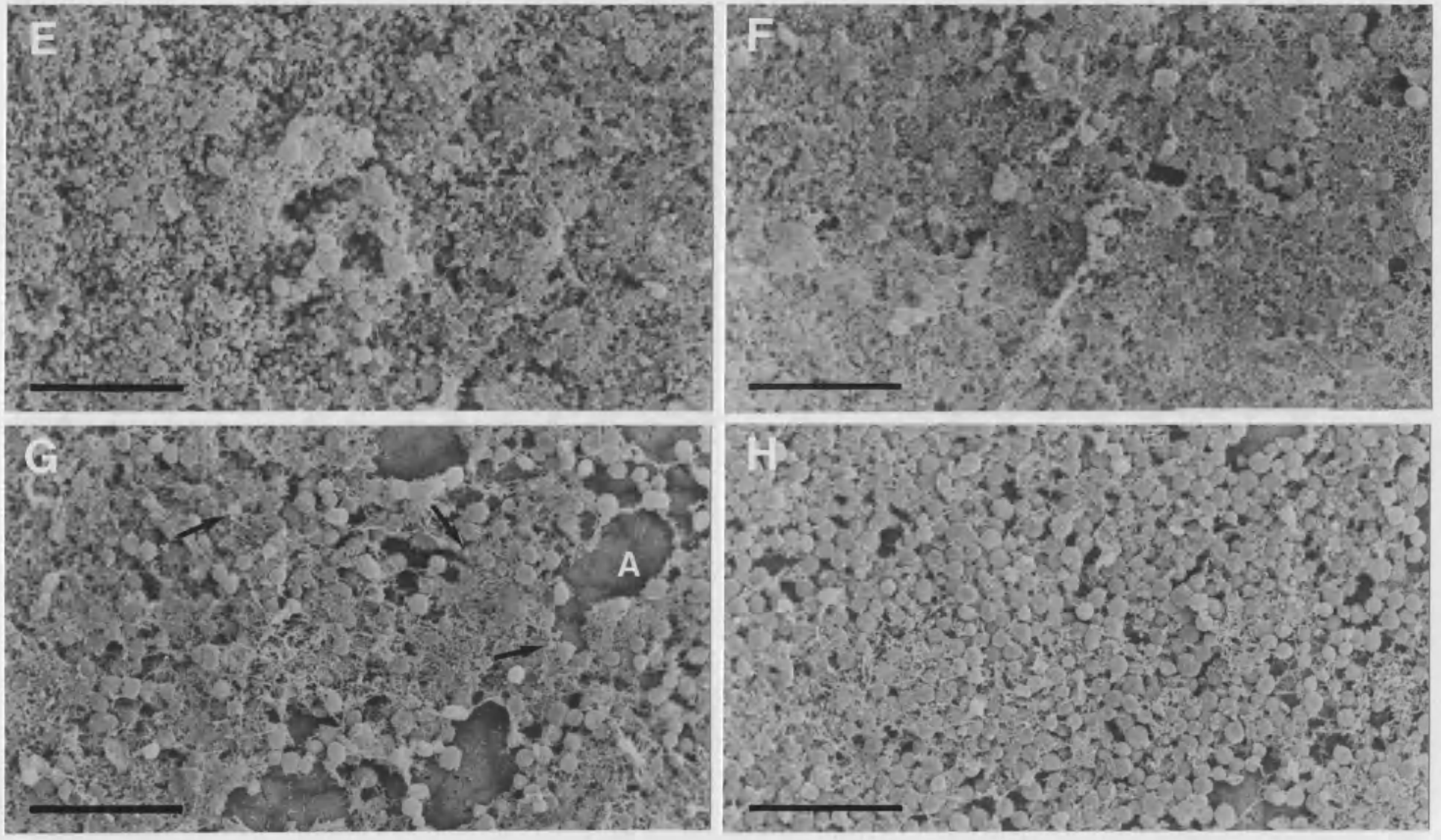


Fig. 3.2 (cont.)



preservation did not allow differentiation between trophozoites and cysts, but complementary light microscopic observations of similar lawns revealed that the amoebae at the plaque edge (Fig.3.2 (E)) were almost exclusively trophozoites, and that the proportion of cysts steadily increased with distance inwards from the plaque edge. The amoebae observed covering the agar surface 2.5mm from the plaque edge (Fig.3.2 (H)) were therefore almost all in the cystic form. Virtually no Microcystis cells remained within the cleared area of the plaque.

(3) Prey range of cyanobacteria

The ability of nine Acanthamoeba strains to predate a range of cyanobacterial species in lawn culture is shown in Table 3.2. Plates were scored either positive or negative for plaque formation after 7d incubation at 30°C. All of the amoebal strains tested were referable to A. castellanii with the exception of 1501/3a and 1501/3b (both A. polyphaga). Most of the amoebae were capable of plaque formation on lawns of most of the prey species tested. Both unicellular (e.g. Synechococcus leopoliensis, Anacystis montana and Microcystis aeruginosa) and filamentous cyanobacteria (e.g. Anabaena spp.) were predated. Two cyanobacteria, Anabaena cylindrica and Tolypothrix tenuis, failed to support the development of plaques with any of the Acanthamoeba strains.

Predation in liquid culture

Tests were made of the ability of ten Acanthamoeba strains (the nine strains listed in Table 3.2 together with A. palestinensis 1547/1) to predate a range of cyanobacteria in liquid culture. The

Table 3.2. The ability of Acanthamoeba to prey on a range of cyanobacteria in lawn culture. (7days incubation at 30°C).

Cyanobacterial species	Acanthamoeba strain								
	BATH PB	NEFF G	BATH 31	CCAP 1501/1a	CCAP 1501/2a	CCAP 1534/2	CCAP 1534/3	CCAP 1501/3a	CCAP 1501/3b
Anabaena A4	+	+	+	-	-	+	+	-	-
Anabaena catenula 1403/1	+	+	+	-	+	+	+	+	+
A. cylindrica 1403/2a	0	0	0	0	0	0	0	0	0
A. variabilis CARR	+	+	+	-	+	+	+	+	+
Anacystis montana 1430/1	+	+	+	+	+	+	+	+	+
An. nidulans CARR	+	+	+	-	-	+	+	-	-
Microcystis aeruginosa 1450/1	+	+	+	0	+	+	+	+	+
Plectonema boryanum Daft 594	+	+	+	0	+	+	+	+	+
Synechococcus leopoliensis 1405/1	+	+	+	+	+	0	+	+	+
Tolypothrix tenuis 1482/3a	-	0	0	0	0	-	0	-	-

+ = Plaque 0 = No plaque - = Not tested

cyanobacterial strains used were Synechococcus leopoliensis, Anacystis nidulans, Anabaena A4, A. variabilis, A. cylindrica CARR, A. catenula, Nostoc muscorum WHIT, Calothrix parietina, Aphanizomenon flos-aquae, Plectonema boryanum, Spirulina sp. 13-TE and Spirulina sp. S24 (both Spirulina strains were resuspended in Allen's medium since the high alkalinity of their normal growth medium prevented amoebal predation).

Microscopic observations of cyanobacterial/amoebal mixtures after 7d incubation showed that all cyanobacterial strains tested, with the exception of Calothrix parietina, Nostoc muscorum, and Anabaena cylindrica (which were not predated by any of the acanthamoebae), were predated by all the Acanthamoeba strains tested. The cyanobacteria most susceptible to predation, measured in terms of the degree of clearance achieved in the Repli dish wells, were Synechococcus leopoliensis, Anacystis nidulans, Anabaena variabilis, Anabaena A4 and Plectonema boryanum. Although all the cyanobacteria that were predated were ingested by all the Acanthamoeba strains, the voracity with which the cyanobacteria were predated (assessed in terms of the extent of clearance of cyanobacteria in the Repli dish wells) varied considerably amongst the Acanthamoeba strains tested. Strain numbers 31, PB, 1534/2, G and 1534/3 were consistently the most effective predators. Strain numbers 1501/1a and 1547/1 were consistently poor predators.

Discussion

The results of the predation trials in both lawn and liquid culture demonstrate that strains of *Acanthamoeba castellanii* and *A. polyphaga* are both capable of utilizing a wide range of cyanobacterial species. *A. palestinensis* (studied in liquid culture only) was a less effective predator. The observed variation in the voracity with which different amoebal strains consumed cyanobacteria could not be explained by disparities in the sizes of trophozoites. The mean diameter of rounded trophozoites of all ten strains was very similar at between 18 and 26um (Table 3.1). Differences in voracity were more likely caused by differences in amoebal growth rates, although comparisons of these were not made. Cyanobacteria were consumed irrespective of their size, shape or growth habit. Those species which were not utilized (*N. muscorum*, *A. cylindrica*, *Tolypothrix tenuis* and *Calothrix parietina*) all produced large quantities of mucilage, and this may have been the basis of their unsuitability as prey. The possession of copious mucilage may possibly confer resistance to predation by providing a physical barrier between the amoeba and its intended prey. The inability of *Acanthamoeba* to consume *Anabaena cylindrica* and *Nostoc muscorum* has also been reported by Wright *et al* (1981). Huang & Wu (1982) found that amoebal isolates from Paddy fields were unable to consume cyanobacteria which had distinct mucilaginous sheaths.

Plaque formation on cyanobacterial lawns due to amoebal predation has previously been reported by Redhead & Wright (1978), Wright *et al* (1981), Huang & Wu (1982) and Yamamoto (1981).

The observation of lawn cultures by scanning electron

microscopy was valuable in that it complemented the information on plaque formation derived from the light microscopic study of scrapings taken from lawn cultures. It had an added advantage in that it allowed the direct visualization of the distribution and relative abundance of predator and prey over the surface of the lawn. Although there was evidence of some shrinkage of specimens during preparation (prepared blocks were smaller than those freshly excised from the lawn), which would have affected the accuracy of the measurements on either side of the plaque boundary, this did not seriously detract from the validity of results. The high concentration of trophozoites at the plaque boundary, and minimal penetration of trophozoites into the ungrazed lawn, provides an explanation for the concentric increase in diameter of plaques. The occurrence of large-scale encystment of amoebae in the centre of plaques was probably a response to the depletion of the food source in this zone. However, food shortage is only one of a number of factors which are thought to induce encystment in protozoa (see Van Wagtendonk, 1955).

SECTION TWO

QUANTIFICATION OF INGESTION/PREDATION OF CYANOBACTERIA IN MIXED CULTURES

Introduction

One of the main determining factors influencing the predation rate of Acanthamoeba on its cyanobacterial prey is the rate at which food particles are ingested at the cell surface. In an attempt to study this aspect of predation, experiments were designed to enable quantification of the uptake of a suitable cyanobacterial food source by Acanthamoeba.

There are a number of ways of assessing the extent of cyanobacterial ingestion by Acanthamoeba, the most desirable of which would be to record the mean number of ingested cells per amoeba after a given incubation time. However, a major drawback in this is the difficulty of accurately counting individual cyanobacterial cells within the amoebae, especially once digestion of the food has commenced. For this reason it was decided to quantify ingestion by recording the percentage of the amoebal population which contained ingested cyanobacterial cells, in whatever number, after various periods of incubation. Using this system a comparison was made of the rate of ingestion of cyanobacterial cells by amoebae resuspended in Allen's medium, and by those suspended in their own (PGY) growth medium. In addition, experiments were conducted to determine the effect of incubation temperature on cyanobacterial uptake. The enumeration of amoebal and cyanobacterial populations at different stages of incubation allowed investigation of the relationship between

the rate of ingestion of food particles and its effect on amoebal proliferation and cyanobacterial decline.

This section also investigates two other aspects of amoebal predation. Firstly, a comparison was made of the predation of three cyanobacterial species by a single amoebal strain, Acanthamoeba castellanii PB. The aim of this was to provide quantitative information to determine whether different food organisms were consumed at different rates, and whether they supported different degrees of amoebal proliferation. Secondly, experiments were set up to investigate the influence of pH on the ability of Acanthamoeba to predate cyanobacteria. Populations of predator and prey were suspended together in buffers of different pH, and cell counts on samples taken after various periods of incubation permitted quantitative measurement of the extent to which pH affected predation.

In most of the studies in this section, and indeed in succeeding sections, Acanthamoeba castellanii PB and Anabaena A4 were selected as the predator and prey organisms. The rationale behind this choice was as follows: A. castellanii PB was selected on the basis that it grew well in batch culture and fed voraciously on a wide range of cyanobacteria in both lawn and liquid culture (see Section one). Anabaena A4 was selected because of its susceptibility to predation (Section one) and also because it grew as a homogenous suspension in liquid culture. This latter factor was important in order to attain accurate values for the cyanobacterial population from cell counts or chlorophyll extractions (see Section three).

Materials and Methods.

(1) Ingestion/predation of cyanobacteria in mixed cultures

Preparation of mixed cultures of predator and prey

Samples (1ml) from a 10d batch culture of Anabaena A4 were dispensed into the wells of a divided Repli dish. A suspension of Acanthamoeba cells (A. castellanii strain PB)) was prepared by centrifuging (10min at 500g) 5ml from a 2d batch culture in PGY medium, removing the supernatant, and resuspending the cells in 5ml Allen's medium. This suspension was then diluted with 100ml Allen's medium in a 250ml Erlenmeyer flask, the latter being agitated gently to ensure even distribution of amoebal cells.. 1ml of the diluted suspension was dispensed into the Repli dish wells containing the cyanobacterial suspension, giving a working volume of 2ml. In other cases, where amoebae were added suspended in PGY medium, 2.5ml from a 2d amoebal batch culture were transferred directly to 50ml sterile PGY medium. 1ml of the diluted suspension was added to the wells containing cyanobacteria. Control wells contained 1ml of cyanobacterial suspension and 1ml of either sterile Allen's medium or PGY medium. The contents of each well were thoroughly mixed, by gentle use of a Pasteur pipette, immediately following the introduction of amoebae. Dishes were incubated in the dark at 20°C.

Assessment of ingestion of cyanobacterial cells by amoebae

Samples were fixed after 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 20, 32 and 77h incubation. A destructive sampling regime was employed in which entire well contents were fixed by addition of 1ml 6% (v/v) glutaraldehyde (giving a final concentration of 2%). Two wells of each

treatment were fixed for each sample. The contents of each well were mixed as previously described to ensure rapid immobilization of cells. The experiment was designed in such a way that all the samples to be fixed at a particular time were grouped together in a single Repli dish, thereby removing the possibility of cross-contamination between fixed and non-fixed cells by glutaraldehyde vapour. Dishes of fixed samples were sealed with tape and stored at room temperature prior to microscopic examination of the well contents. Amoebae were scored according to whether or not they contained ingested cyanobacteria. Two hundred amoebae were examined from each sample (100 from each well). The procedure used when scoring was as follows: The well contents were mixed, and a large drop of fluid containing suspended cells placed on a slide with coverslip. The slide was scanned horizontally, and each amoeba entering the field of view was scored for the presence or absence of ingested cyanobacterial cells. After 50 amoebae had been scored the slide preparation was replaced and the process repeated. This method allowed the scoring of a random sample of amoebae.

Quantification of predator and prey populations

Both amoebal and cyanobacterial numbers were determined by haemocytometer counts. The (fixed) contents of the wells were mixed and the volume re-adjusted to 3ml with distilled water (to compensate for evaporative loss during incubation) prior to the withdrawal of cell suspensions for counting. For each sample, four counts (two from each well) were made of the amoebal population and six counts (three from each well) of the cyanobacterial population. Where necessary, samples were diluted to facilitate counting.

The effect of temperature on ingestion of cyanobacteria

Mixed cultures of predator and prey were prepared as described above (amoebal suspensions were prepared in Allen's medium). Cultures were incubated in the dark at 4°C, 10°C, 20°C and 30°C. The amoebal and cyanobacterial suspensions were left to equilibrate for 1h at their respective incubation temperatures prior to mixing. To facilitate this process, four identical amoebal suspensions were prepared, one for each temperature. Samples were fixed after 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 19, 29, 48, 72, 96 and 120h. The methods used to assess the ingestion of cyanobacteria by Acanthamoeba and to quantify the numbers of predator and prey were the same as those described above.

(2) Comparison of the predation of three cyanobacterial species

The methodology used was similar to that described above. Three cyanobacterial species were used as prey; Anabaena A4, Anabaena variabilis 7118DM and Synechococcus leopoliensis 1405/1. Batch cultures (12d) were used as the inoculum. The density of the cyanobacterial inoculum was standardized for the three species on the basis of optical density (each culture was diluted with sterile Allen's medium to give an OD_{680} of 0.43). Samples (1ml) of each cyanobacterial suspension were dispensed into Repli dish wells, to which were added an equal volume of Acanthamoeba castellanii PB inoculum (prepared by resuspension of cells from a 2d batch culture into Allen's medium). Two amoebal inoculum densities were used, 7.8×10^3 and 45×10^3 cells/ml. Wells containing each prey species were

inoculated with both densities of Acanthamoeba. Control wells received 1ml sterile Allen's medium in place of the amoebal suspension. Dishes were incubated in the dark (to prevent cyanobacterial proliferation) at 20°C. Samples were fixed in glutaraldehyde after 0, 8, 18, 28, 45, 69, 93, 117, 165 and 357h incubation. At each sampling two wells of each cyanobacterium were fixed for each of the amoebal inoculum levels. Haemocytometer counts were used to enumerate both the cyanobacterial (three counts per well) and amoebal (two counts per well) populations.

(3) The Effect of pH on predation

Separate suspensions of cyanobacteria, and of amoebae, were prepared in buffer at each of the pH values to be tested. Citrate phosphate buffer was used for pH 4.0, 5.0, 5.6, 6.0, 6.6 and 7.0, and Tris buffer for pH 7.2, 7.6, 8.0, 8.6 and 9.0 (see Appendix B for composition). Each cyanobacterial suspension was prepared by centrifugation (10min, 1300g) of two samples (5ml) from a 21d batch culture of Anabaena A4, removal of the supernatant, and resuspension of each sample of cells in 6ml buffer of the desired pH. 1ml of this suspension was dispensed into 10 Repli dish wells. Suspensions of acanthamoebae were prepared by centrifuging (10min, 500g) samples (4ml) from a 2d batch culture, removing the supernatant, and resuspending the cells in 4ml buffer of the appropriate pH. This suspension was then diluted with 100ml of the same buffer in a 250ml Erlenmeyer flask. 1ml of the diluted suspension was added to those wells containing the cyanobacteria (suspended in buffer at the same pH), giving a final volume of 2ml. In control wells the amoebal

suspension was replaced by an equal volume of buffer of the appropriate pH. The contents of each well were thoroughly mixed at the time of inoculation. Two sets of five wells (three experimental, two control) were set up for each pH value. Dishes were incubated in the dark at 20°C.

Complete sets of wells were fixed after 4d and 8d incubation. The contents of each well were inactivated by addition of 1ml 6% (v/v) glutaraldehyde. Amoebal and cyanobacterial numbers were determined by haemocytometer counts (two counts per well for Acanthamoeba, three counts per well for Anabaena).

Results

(1) Ingestion/predation of cyanobacteria in mixed cultures

Amoebae were scored positively for ingestion if they were in the process of ingesting an Anabaena filament or if they contained recognizeable Anabaena cells, the latter being much the more frequent observation. Beyond a certain stage in the digestive process the cyanobacterial cells became indistinguishable as discrete entities, forming instead an amorphous green-brown mass. Amoebae which only contained cyanobacteria in this condition were negatively scored.

The rate at which cyanobacteria were ingested by Acanthamoeba, measured in terms of the percentage of the amoebal population containing ingested cells (hereafter referred to as percentage ingestion), at twelve sampling times ranging from 0.25 to 77h after introduction of the food source, is presented in Table 4.1 and Fig.4.1 (only values for first 8h plotted). The pattern of ingestion is similar for both treatments (those in which the amoebal inocula were

Fig.4.1. The percentage of the A. castellanii population containing Anabaena A4 cells (percentage ingestion) at various times after introduction of the food source.
Dark incubated, 20°C. (Amoebal inocula for the Allen's and PGY treatments were resuspended in Allen's and PGY medium respectively).

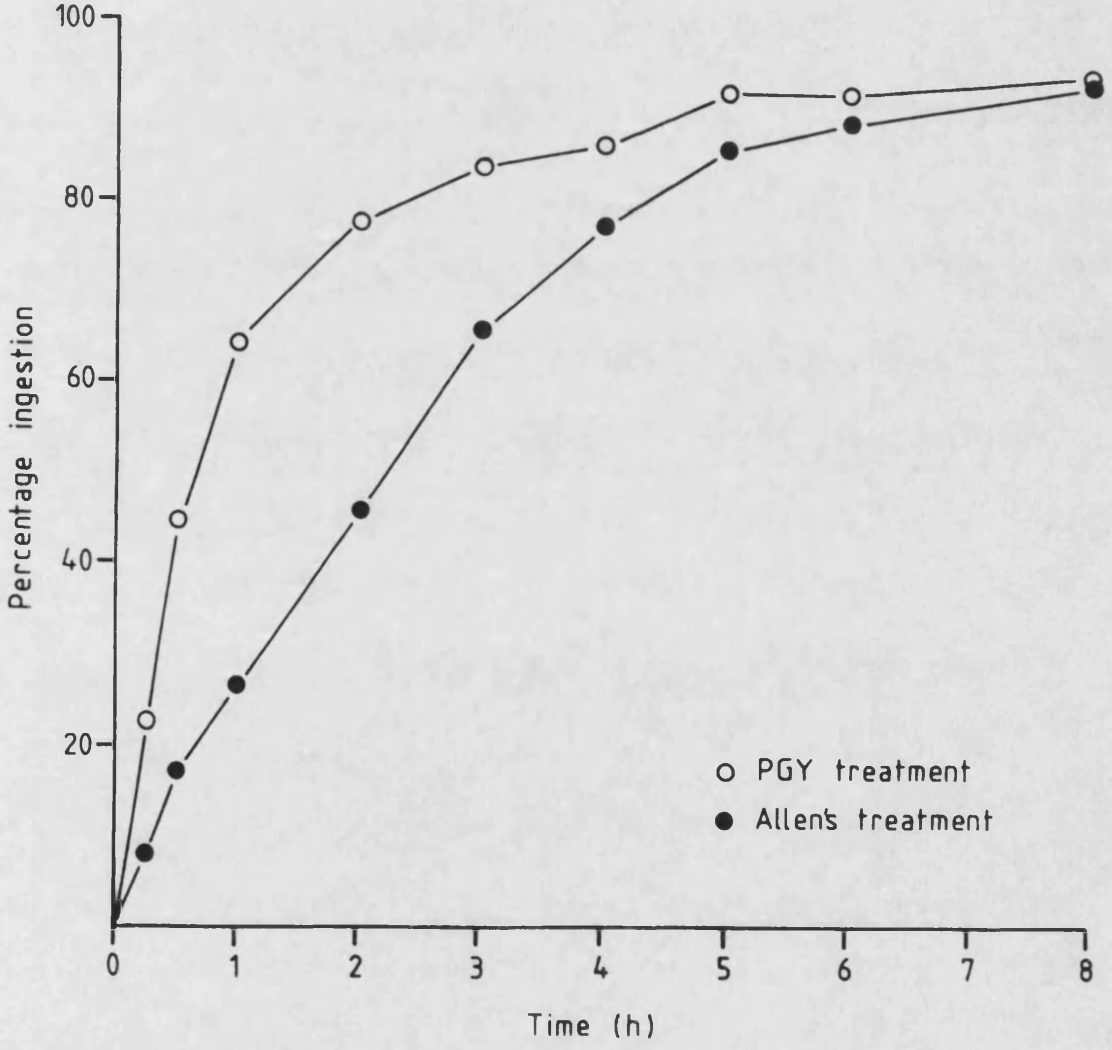


Table 4.1. The percentage of the A. castellanii PB population containing ingested Anabaena A4 cells, at different times after introduction of the food source, during incubation in the dark at 20°C.

Time (h)	% of A. castellanii population ingesting cyanobacteria*	
	Allen's treatment+	PGY treatment+
0.25	8.0 (1.8)	22.5 (1.7)
0.5	17.0 (2.1)	44.5 (3.8)
1	26.5 (2.2)	64.0 (2.3)
2	45.5 (1.5)	77.5 (2.2)
3	65.5 (5.3)	83.5 (2.2)
4	77.0 (3.9)	86.0 (2.4)
5	85.5 (3.3)	92.0 (0.8)
6	88.5 (2.5)	91.5 (2.2)
8	92.5 (3.1)	93.5 (2.2)
20	80.0 (1.7)	82.0 (2.6)
32	78.5 (1.5)	58.0 (4.2)
77	9.0 (1.9)	5.5 (2.1)

* Each value was derived from the scoring of 200 trophozoites. Figures in parenthesis are +/- one standard error.

+ Amoebal inocula for the Allen's and PGY treatments were resuspended in Allen's and PGY medium, respectively.

resuspended in Allen's medium, "Allen's treatment", and those in which the amoebal inocula were suspended in their own growth medium, "PGY treatment"). In each case the onset of ingestion occurred soon after introduction of the food source, with percentage ingestion increasing rapidly with successive samplings, so that after 8h incubation over 90% of amoebae in both treatments contained ingested cyanobacteria.

The two treatments differed in that the rate of uptake in the PGY treatment was greater than for the Allen's treatment. Over the first 8h of incubation, at any given sampling point the percentage ingestion for amoebae in the PGY treatment was always higher than for amoebae in the Allen's treatment. Thus 15min after mixing of predator and prey populations, 22.5% of trophozoites in the PGY treatment contained cyanobacteria as opposed to 8% for the Allen's treatment. The corresponding values for 1h incubation were, respectively, 64% and 26.5%. Reading from Fig.4.1, the time taken for 50% of the amoebal population to contain ingested Anabaena can be estimated as 40min for the PGY treatment and 135min for the Allen's treatment. In both treatments the rate of increase in percentage ingestion declined with time over the initial 8h period, as shown by the flattening out of the curves in Fig.4.1. As the values for percentage ingestion reached a plateau, so the disparity between treatments progressively lessened, being minimal (1%) by 8h incubation. The values for percentage ingestion declined for both treatments at each of the three remaining sampling times (20h, 32h and 77h), the values at 77h being 9% and 5.5% for the Allen's and PGY treatments respectively (Table 4.1).

The increase in percentage ingestion with time was accompanied by increases in the number of cyanobacteria contained within

individual Acanthamoeba cells. Direct microscopic observation of samples showed that after 1h incubation most of those amoebae which had ingested cyanobacteria contained only one or a few cells, whereas after 2h many of the positively scored amoebae contained 10 or 20 Anabaena cells. Qualitative estimates of the number of cyanobacterial cells ingested by each amoeba increased with successive samplings, being maximal after six and eight hours incubation. In these cases the amoebae were bright green in colour and were often so full of cyanobacteria that their cell shape was distorted. Amoebae typically contained a combination of partially ingested filaments, newly ingested cells and older food vacuoles. After 20h incubation amoebae were less densely packed with cyanobacteria than at 8h, and contained many old food vacuoles. In the samples for 32h and 77h the majority of amoebae only contained cyanobacteria in an advanced state of digestion, and so could not be scored as positive for ingestion under the stated criteria (see above). The percentage ingestion for these times was therefore lower than in the preceeding samples (see Table 4.1). After 77h incubation some rounding-up of trophozoites was observed, although no cysts were recorded. At the early sampling times (2h, 3h) amoebae in the PGY treatment contained noticeably more cyanobacteria per cell than in the Allen's treatment, a difference which became less apparent at subsequent samplings.

Counts of the amoebal and cyanobacterial populations at selected samplings are given in Tables 4.2 and 4.3 respectively. The development of predator and prey numbers over the duration of the experiment is shown in Fig. 4.2. In both treatments amoebal predation resulted in a severe reduction of the Anabaena population, numbers

Table 4.2. Changes with incubation time in A. castellanii PB populations presented with Anabaena A4 as a food source. (Cultures were incubated in the dark at 20°C).

Time (h)	A. castellanii Population ($x10^3$ cells/ml)*	
	Allen's Treatment+	PGY Treatment+
0.25	70.6 (2.5)	71.7 (1.3)
4	72.7 (3.0)	73.1 (3.6)
8	80.6 (2.4)	85.2 (4.4)
20	125.2 (5.4)	122.9 (2.8)
32	169.4 (15.4)	157.5 (15.3)
77	200.6 (10.4)	241.3 (12.8)

* Each value is the mean of four counts. Figures in parenthesis are +/- one standard error.

+ Amoebal inocula for the Allen's and PGY treatments were resuspended in Allen's and PGY medium, respectively.

Table 4.3. Decline in Anabaena A4 populations in mixed cultures due to predation by A. castellanii PB.
(Cultures were incubated in the dark at 20°C).

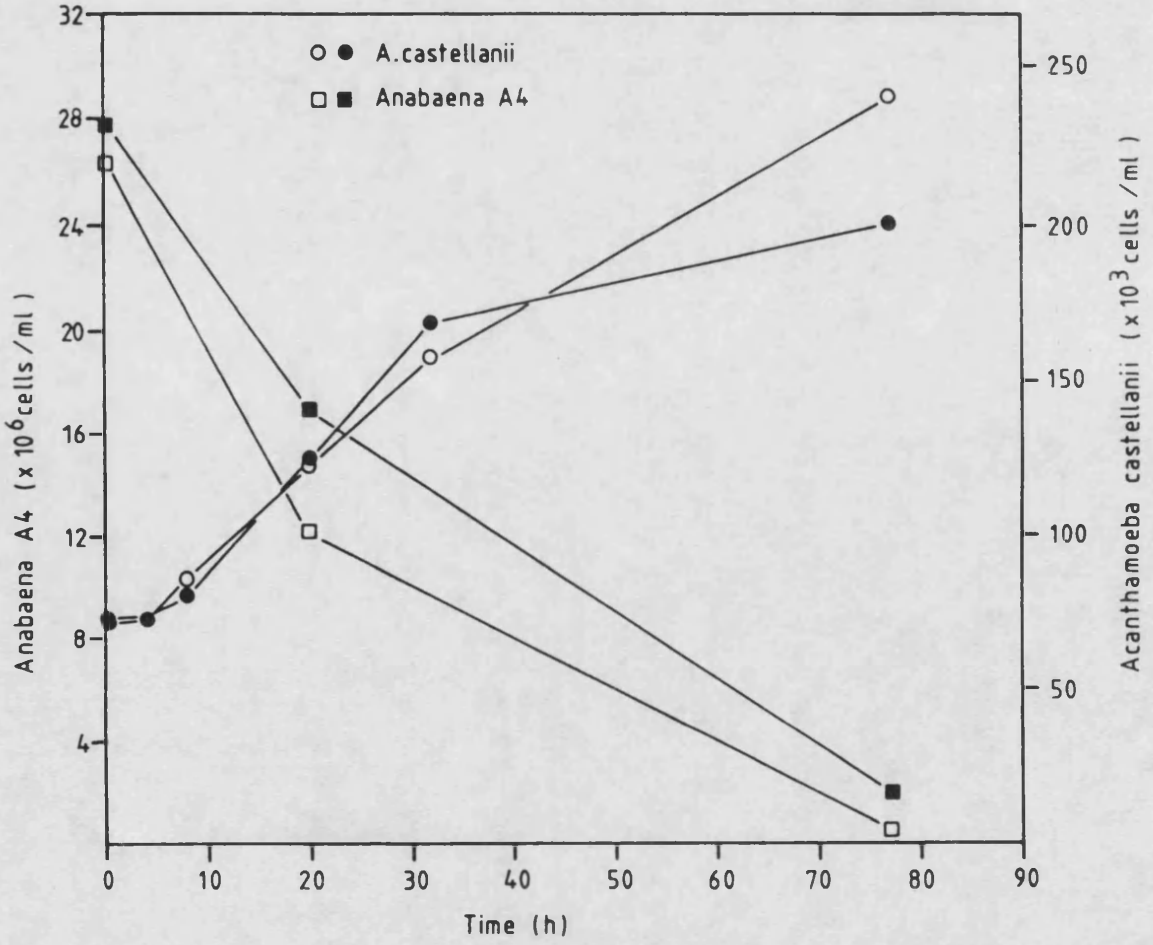
Time (h)	Anabaena population ($x10^6$ cells/ml)*	
	Allen's Treatment+	PGY Treatment+
0.25	27.7 (2.3)	26.4 (2.2)
20	16.8 (1.4)	12.1 (1.0)
77	2.0 (0.3)	0.4 (0.2)

* Each value is the mean of six counts. Figures in parenthesis are +/- one standard error.

+ Amoebal inocula for the Allen's and PGY treatments were resuspended in Allen's and PGY medium, respectively.

Fig.4.2. The change in A. castellanii and Anabaena A4 populations with time in mixed cultures of predator and prey incubated at 20°C.

(Open symbols = PGY treatment, closed symbols = Allen's treatment).



declining after 77h incubation from $27.7x10^6$ to $2.0x10^6$ cells/ml in the Allen's treatment, and from $26.4x10^6$ to $0.4x10^6$ cells/ml in the PGY treatment (Table 4.3). These values represent a decrease in cyanobacterial population of 93% and 98.4% for the Allen's and PGY treatments, respectively. The decline in cyanobacterial population was more rapid in the PGY treatment, as shown by the 54% decrease (over the initial population) in Anabaena after 20h incubation in the PGY wells compared with a 39% decrease in the Allen's wells. No counts were made of the cyanobacterial population in control wells, although visual comparison revealed major differences between the control and experimental wells. In experimental wells there was a progressive clearing of the cyanobacterial growth during incubation, and after 77h virtually no Anabaena cells remained. In contrast, control wells contained a dense, healthy population of Anabaena throughout the duration of the experiment (and despite incubation in the dark), thereby indicating little or no change in cyanobacterial numbers.

The decrease in cyanobacterial population in the experimental wells was accompanied by an increase in numbers of Acanthamoeba. The initial amoebal population was approximately $70x10^3$ cells/ml, and followed a similar pattern of development for both treatments. After 32h incubation amoebal numbers had risen to $169.4x10^3$ cells/ml in the Allen's treatment and $157.5x10^3$ cells/ml in the PGY treatment (Table 4.2), increases of 140% and 120% respectively. The corresponding values for 77h were $200.6x10^3$ cells/ml for the Allen's treatment (184% increase over initial population) and $241.3x10^3$ cells/ml for the PGY treatment (237% increase).

The Effect of Temperature

Incubation temperature had a marked effect on the ingestion of Anabaena A4 cells by A. castellanii. Both the rate of increase in percentage ingestion and the eventual maximum percentage of the amoebal population with ingested Anabaena were affected. Ingestion was greatest at 20 and 30°C, reduced at 10°C and strongly inhibited at 4°C (Fig.4.3, Table 4.4). The pattern of uptake of Anabaena A4 cells was very similar at 20 and 30°C, with an initial rapid phase (90.5% ingestion for both temperatures after 2h incubation). Percentage ingestion increased slowly at subsequent samplings, attaining a maximum of 97% after 5h at 30°C, and 98% after 6h at 20°C. At 10°C, 69.5% of the amoebal population had ingested Anabaena cells after 2h. Although increasing in subsequent samples, percentage ingestion did not attain the level recorded for the two higher temperatures, the maximum value being 88% after 19h. At 4°C only 6.5% of amoebae had ingested cyanobacteria after 2h incubation, values fluctuating around this level in subsequent samples. A maximum of 18% ingestion was recorded after 19h incubation (Table 4.4). Qualitative observations of fixed amoebae at each of the four temperatures showed that the number of ingested Anabaena cells per positively scored amoeba was far fewer at 4°C than at the other temperatures for all sampling times with the exception of those where the cyanobacterial population had been virtually eliminated by predation (i.e. after 72, 96 and 120h incubation at 20°C and 30°C - Table 4.5).

The differences in cyanobacterial ingestion at the four incubation temperatures were reflected in the extent and rate of disappearance of Anabaena A4 from the Repli dish wells over the 5d of

Fig.4.3. The effect of temperature on the percentage of the A. castellanii population containing Anabaena A4 cells (percentage ingestion) at various times after introduction of the food source.

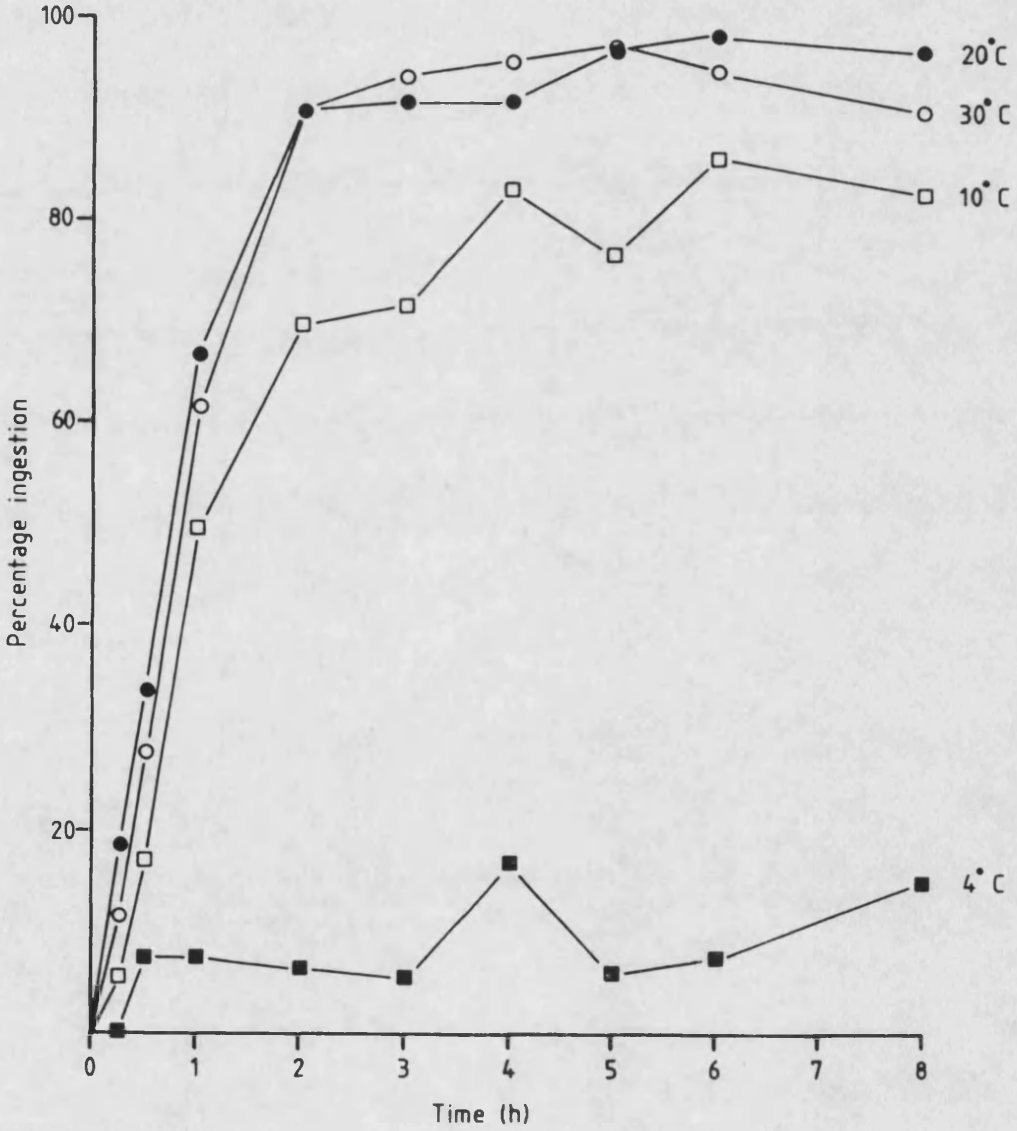


Table 4.4. The influence of incubation temperature on the percentage of the Acanthamoeba castellanii PB population with ingested Anabaena A4 at various times after introduction of the food source.

Time (h)	% of A. castellanii population ingesting cyanobacteria*			
	4°C	10°C	20°C	30°C
0.25	0 -	5.5 (1.5)	18.5 (2.2)	11.5 (1.7)
0.5	7.5 (2.5)	17.0 (1.3)	33.5 (2.6)	27.5 (1.7)
1	7.5 (3.6)	49.5 (2.9)	66.5 (1.7)	61.5 (4.6)
2	6.5 (2.1)	69.5 (2.2)	90.5 (2.9)	90.5 (1.5)
3	5.4 (1.0)	71.5 (4.3)	91.5 (2.9)	94.0 (0.8)
4	17.0 (4.4)	83.0 (1.7)	91.5 (1.5)	94.5 (1.7)
5	6.0 (2.0)	76.5 (3.6)	96.5 (1.3)	97.0 (1.0)
6	7.5 (1.0)	86.0 (2.2)	98.0 (1.2)	94.5 (1.9)
8	15.0 (4.8)	82.5 (3.4)	96.5 (1.0)	90.5 (2.4)
19	18.0 (2.4)	88.0 (2.8)	92.5 (1.7)	75.0 (3.4)

* Each value was derived from the scoring of 200 amoebae. Figures in parenthesis are +/- one standard error.

Fig.4.4. The effect of temperature on the proliferation of A. castellanii with Anabaena A4 as the food source.

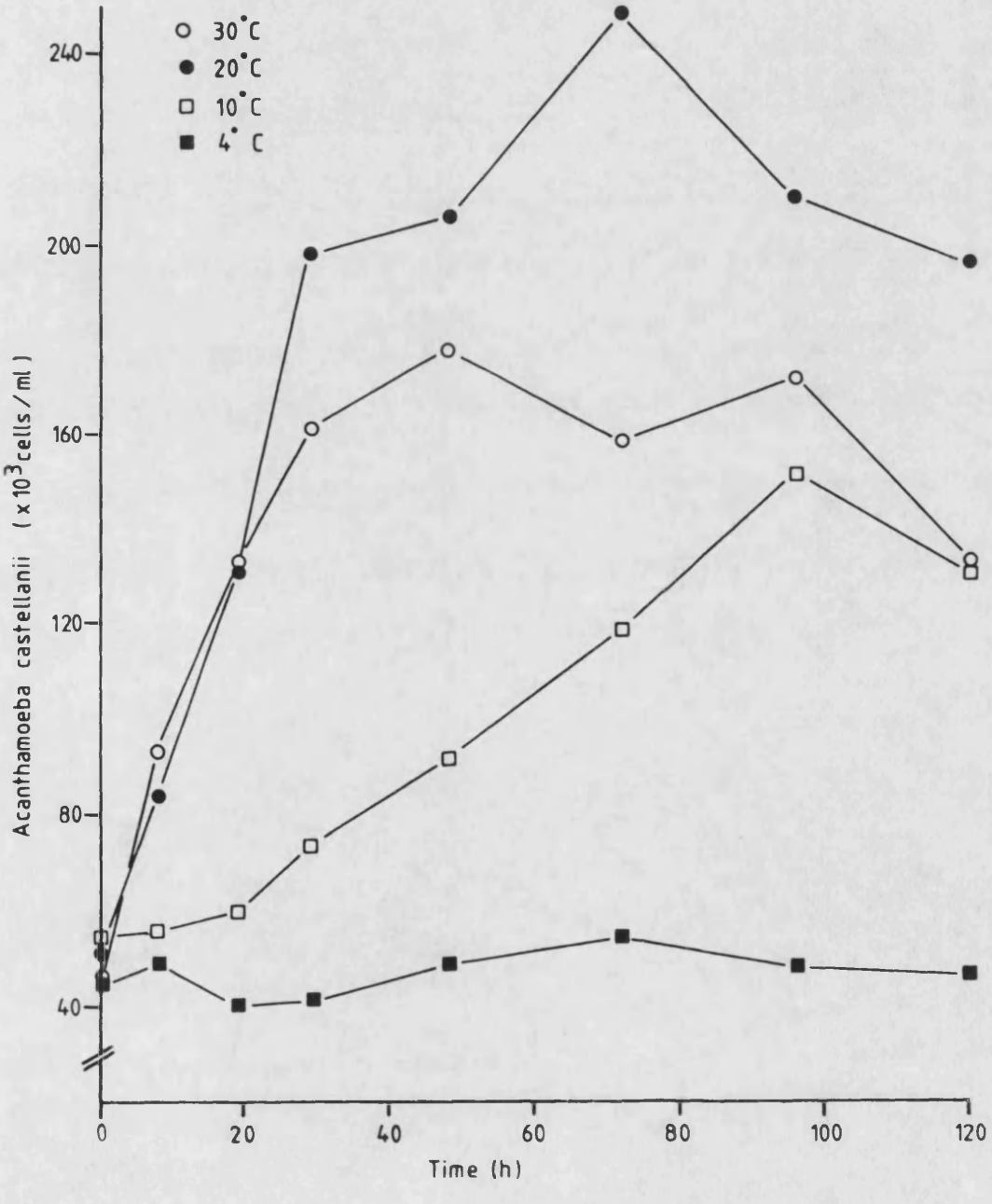


Table 4.5. The influence of temperature on the predation of Anabaena A4 by Acanthamoeba castellanii PB: Changes in cyanobacterial populations over five days incubation.

Sample	Time (h)	Anabaena A4 population ($x10^6$ cells/ml)*			
		4°C	10°C	20°C	30°C
Experimental	0.25	27.6 (3.0)	27.5 (2.3)	26.7 (0.9)	26.8 (1.8)
"	8	24.4 (2.3)	25.6 (0.8)	20.3 (1.7)	15.2 (1.2)
"	19	29.2 (2.0)	22.9 (1.5)	13.3 (1.0)	9.7 (0.8)
"	29	26.8 (1.1)	22.5 (2.3)	5.6 (0.4)	3.1 (0.3)
"	48	27.0 (1.5)	16.2 (0.9)	1.4 (0)	1.5 (0.2)
"	72	29.7 (0.6)	16.7 (0.7)	0.2 (0)	0.1 (0)
"	96	28.2 (2.0)	8.8 (0.7)	0 (0)	0.1 (0)
"	120	26.2 (1.0)	11.2 (1.4)	0.1 (0.1)	0.1 (0)
Controls	120	27.9 (2.7)	29.1 (1.2)	32.9 (0.8)	28.5 (1.5)

* Each value is the mean of six counts. Figures in parenthesis are +/- one standard error.

the experiment (Table 4.5, Fig. 4.5). Counts of Anabaena after 0.25h incubation were taken to be the initial population, since very few cyanobacterial cells were ingested in the period between the mixing of predator and prey and fixing of the first sample. The decline in cyanobacterial population was greatest at 20 and 30°C, negligable at 4°C and intermediate at 10°C. At 4°C the Anabaena population remained at approximately $27x10^6$ cells/ml throughout the experiment. After 19h cyanobacterial numbers had increased by 7% at 4°C and declined by 16%, 51% and 64% at 10, 20 and 30°C respectively in comparison with the initial population (mean value for the four treatments combined). After 120h incubation, numbers had decreased by 4%, 59%, 99.6% and 99.8% compared with their initial levels at 4, 10, 20 and 30°C respectively. Thus at the two higher temperatures less than 1% of the initial Anabaena population remained after 5d. In all cases control wells showed no visible reduction in the Anabaena population, counts for those at the 120h sample instead revealing an increase in cell numbers (see Table 4.5).

The development of the amoebal population at each of the four temperatures is shown in Table 4.6 and Fig.4.4. At 4°C the Acanthamoeba population remained virtually unchanged throughout the experiment. At 10°C amoebal numbers increased steadily with incubation time, reaching a peak of $151x10^3$ cells/ml (208% increase over the mean of the initial populations for the four temperatures combined) after 96h before declining slightly to $131x10^3$ cells/ml after 120h. At both 20°C and 30°C there was a sharp increase in the first 29h after mixing, amoebal numbers rising to $198x10^3$ cells/ml at 20°C (304% increase) and $161x10^3$ cells/ml at 30°C (227% increase). Thereafter

Fig.4.5. The effect of temperature on the predation of Anabaena A4 by A. castellanii.

(Amoebal inoculum ≙ $50x10^3$ cells/ml).

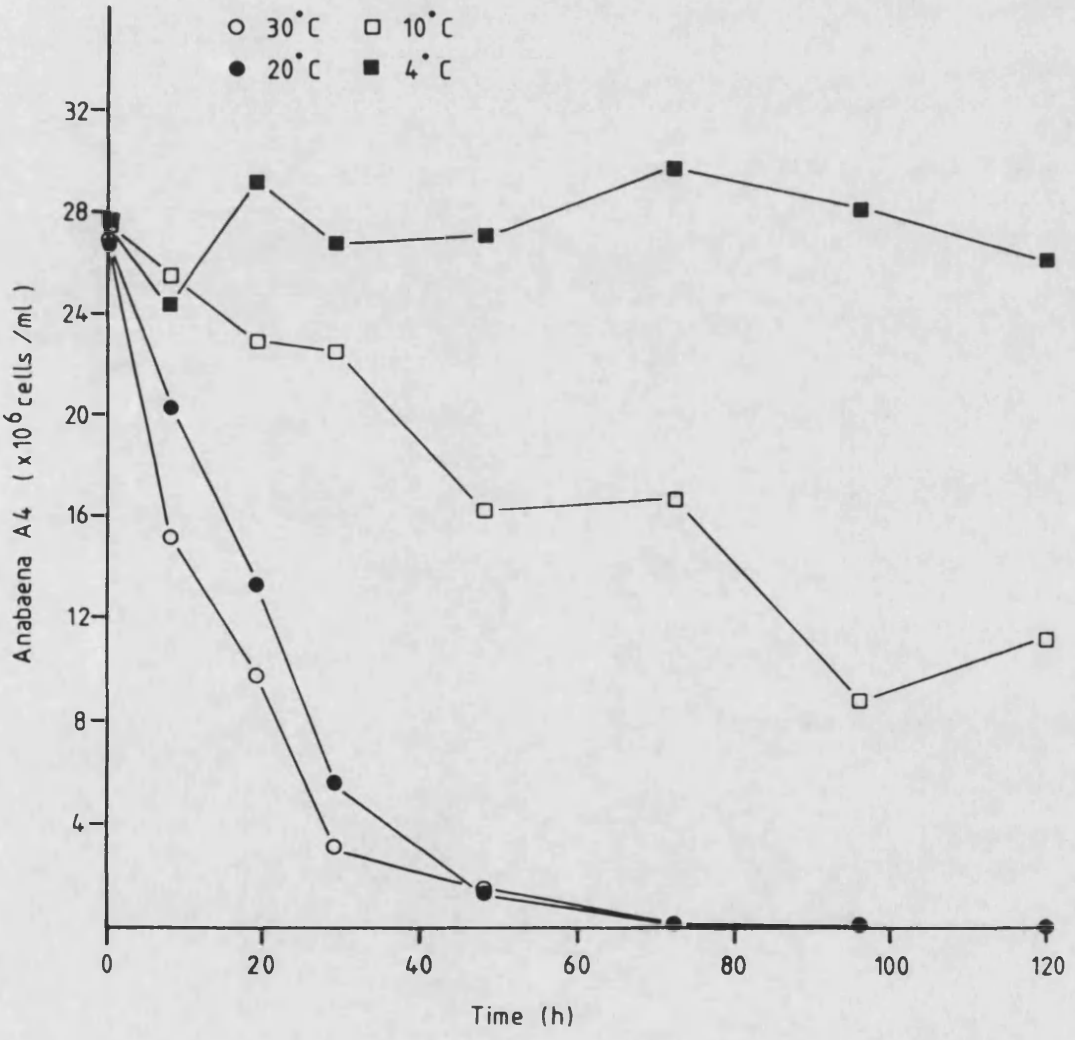


Table 4.6. The influence of temperature on the predation of Anabaena A4 by Acanthamoeba castellanii PB. Changes in total amoebal count (TAC), and the percentage of the amoebal population present as cysts (%E), over five days incubation.

Time (h)	Acanthamoeba castellanii population ($x10^3$ cells/ml)*							
	4°C		10°C		20°C		30°C	
	TAC	%E	TAC	%E	TAC	%E	TAC	%E
0.25	44.8 (2.1)	0	54.2 (2.2)	0	51.0 (4.0)	0	46.3 (3.4)	0
8	49.2 (1.0)	0	55.6 (4.8)	0	83.8 (3.4)	0	93.1 (6.7)	0
19	40.4 (4.2)	0	59.8 (1.6)	0	130.4 (4.1)	0	132.7 (6.4)	0
29	41.7 (1.0)	0	73.1 (4.6)	0	198.1 (5.1)	0	160.6 (13.7)	0
48	48.8 (1.4)	0	91.5 (8.1)	0	205.8 (5.0)	0	177.5 (13.8)	3.3
72	54.8 (2.2)	0	118.5 (2.8)	0	248.3 (14.4)	7.4	158.3 (14.2)	12.1
96	48.5 (2.9)	0	151.3 (8.9)	0	210.0 (17.6)	11.1	171.7 (21.0)	19.4
120	46.7 (2.0)	0	130.6 (8.3)	0	196.7 (5.3)	10.6	133.3 (6.9)	16.3

numbers remained stable at 30°C until 96h, but declined to $133x10^3$ cells/ml after 120h. At 20°C Acanthamoeba further increased to reach a peak of $248x10^3$ cells/ml at 72h before dropping to $197x10^3$ cells/ml after 120h.

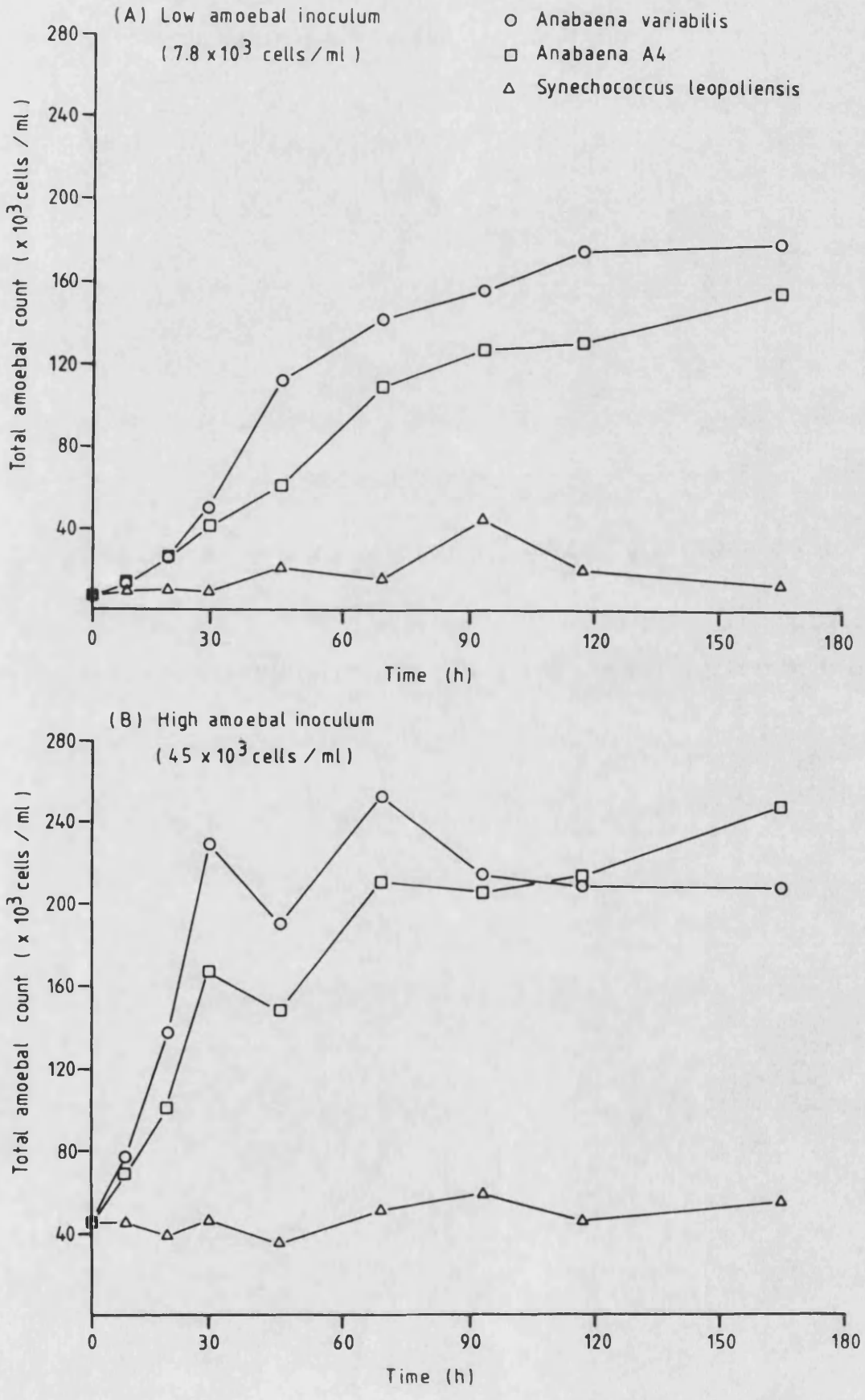
Some differences were noted in the relationship between the amoebal and cyanobacterial populations at each of the four temperatures. At both 20°C and 30°C there was an immediate and rapid increase in the amoebal population consequent upon the predation of Anabaena cells. In contrast, at 10°C there was a slight lag before the ingestion of cyanobacteria was reflected in any large increase in the numbers of Acanthamoeba present. At 4°C there was no effective interaction, with both predator and prey populations remaining stable throughout the experiment.

Encystment of Acanthamoeba was recorded at the two higher temperatures, but not at 4°C or 10°C, during the latter stages of incubation. Cysts were first observed after 48h at 30°C and after 72h at 20°C. After 120h, the proportion of the amoebal population present as cysts was 10.6% at 20°C and 16.3% at 30°C (Table 4.6).

(2) Comparison of the predation of three cyanobacterial species

The rate and extent of proliferation of Acanthamoeba castellanii strain PB upon the three cyanobacterial species is shown in Fig.4.6. The observed pattern of amoebal proliferation was similar for both levels of amoebal inoculum, with the two Anabaena spp. supporting large increases in numbers of acanthamoebae. In both cases the maximal amoebal population was obtained with A. variabilis as the food source. In contrast to the Anabaena spp., Synechococcus

Fig.4.6. Proliferation of Acanthamoeba castellanii PB upon three species of cyanobacteria (20°C, dark incubated).



leopoliensis only supported a relatively small increase in amoebal numbers. The maximum amoebal counts for wells with the low amoebal inoculum were $155x10^3$ cells/ml (after 165h incubation) with Anabaena A4 as the food source, $178.8x10^3$ cells/ml with A. variabilis (165h) and $44.6x10^3$ cells/ml with S. leopoliensis (93h) (Fig.4.6). These were increases of 19.9, 22.9 and 5.7-fold, respectively, over the initial Acanthamoeba population. Maximal populations with the high amoebal inoculum were $246.9x10^3$ cells/ml when fed Anabaena A4 (165h), $251.9x10^3$ cells/ml with A. variabilis (69h) and $67.7x10^3$ cells/ml with S. leopoliensis (357h), representing increases over the initial population of 5.5, 5.6 and 1.5-fold.

The decline in the populations of Anabaena A4 and A. variabilis, for both amoebal inoculum densities, is presented in Fig.4.7. The initial cyanobacterial populations were $29.4x10^6$ cells/ml for Anabaena A4 and $19.3x10^6$ cells/ml for A. variabilis. Enumeration of the Synechococcus population was not possible owing to the small size of the cells. After 117h incubation, predation by Acanthamoeba had virtually eliminated both Anabaena spp. at both amoebal inoculum densities, cyanobacterial numbers having been reduced to less than 0.1% of their initial level. The period of most rapid decrease in cyanobacterial numbers corresponded well with the period of most rapid increase in the amoebal population. For example, with A. variabilis these rapid changes in predator and prey populations occurred between 18 and 45h incubation for the low amoebal inoculum and between 0 and 28h incubation for the high inoculum. The immediate reduction in the population of both Anabaena spp. in the presence of the high amoebal inoculum was in contrast to the low amoebal inoculum, in which there

Fig.4.7. The decline of Anabaena variabilis and Anabaena A4 populations in cultures inoculated with Acanthamoeba castellanii PB.

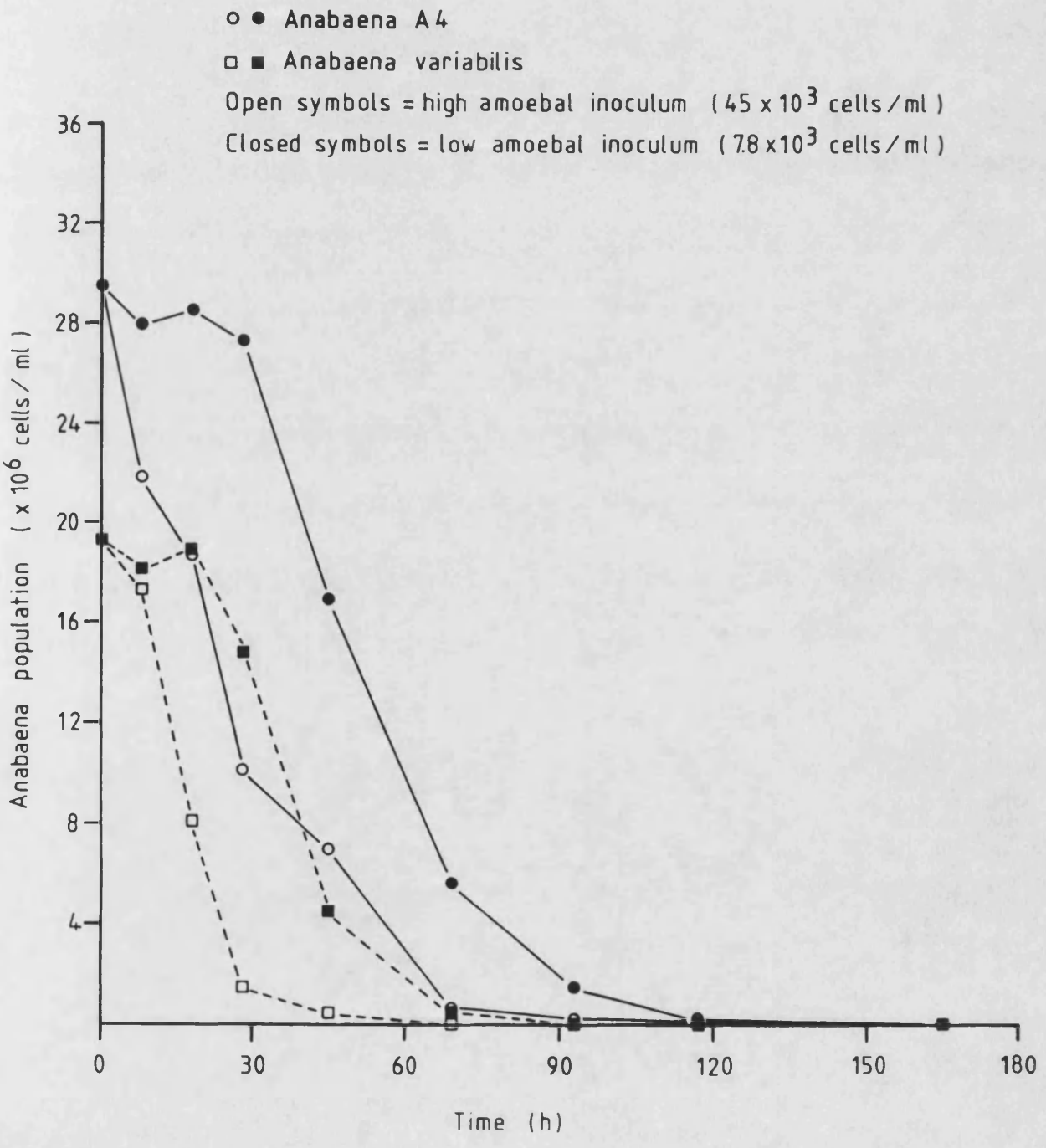


Table 4.7. Comparison of the ability of three cyanobacterial species to support proliferation of Acanthamoeba castellanii PB. Development of trophozoite (T) and cyst (C) numbers, and percentage encystment (%E), for two densities of amoebal inoculum.

(A) Low amoebal inoculum (7.8×10^3 cells/ml)

Time (h)	ACANTHAMOEBA CASTELLANII POPULATION*								
	(Anabaena A4)			(A.variabilis)			(S.leopoliensis)		
	T	C	%E	T	C	%E	T	C	%E
0	7.8 (0.6)	0	0	7.8 (0.6)	0	0	7.8 (0.6)	0	0
8	13.1 (1.8)	0	0	12.5 (1.6)	0	0	9.8 (1.4)	0	0
18	25.8 (2.2)	0	0	24.8 (1.9)	0	0	10.0 (0.9)	0	0
28	44.9 (3.1)	0	0	49.8 (3.7)	0	0	9.4 (0.6)	0	0
45	61.9 (4.9)	0	0	111.9 (3.8)	0	0	21.7 (1.2)	0	0
69	109.4 (8.1)	0	0	141.9 (5.5)	0	0	15.8 (2.2)	0	0
93	117.5 (8.0)	10.0 (1.8)	7.8	140.0 (10.2)	16.3 (3.6)	10.4	44.6 (6.3)	0	0
117	94.4 (3.3)	36.3 (8.8)	27.8	143.8 (12.6)	31.9 (6.9)	18.2	19.6 (3.1)	0	0
165	110.6 (9.2)	44.4 (15.9)	28.6	128.8 (6.5)	50.0 (8.4)	28.0	12.1 (2.4)	0.2 (0.2)	1.6
357	-	-	-	-	-	-	29.8 (0.9)	3.5 (0.7)	10.5

* Values for trophozoite (n=4) and cyst (n=4) populations are $\times 10^3$ cells/ml. Figures in parenthesis are +/- one standard error.

Table 4.7 (continued)

(B) High amoebal inoculum ($45x10^3$ cells/ml)

Time (h)	ACANTHAMOEBA CASTELLANII POPULATION*								
	(Anabaena A4)			(A.variabilis)			(S.leopoliensis)		
	T	C	%E	T	C	%E	T	C	%E
0	45.0 (1.2)	0	0	45.0 (1.2)	0	0	45.0 (1.2)	0	0
8	68.3 (2.9)	0	0	76.7 (6.7)	0	0	45.2 (4.6)	0	0
18	100.6 (4.6)	0	0	137.5 (10.4)	0	0	39.4 (2.1)	0	0
28	166.9 (9.4)	0	0	228.8 (7.8)	0	0	46.0 (3.8)	0	0
45	148.1 (9.9)	0	0	190.0 (6.2)	0	0	35.2 (1.3)	0.6 (0.4)	1.7
69	208.8 (13.6)	1.3 (1.3)	0.6	249.4 (14.5)	2.5 (1.8)	1.0	49.4 (1.1)	2.3 (0.7)	4.4
93	196.9 (14.0)	8.1 (4.8)	4.0	213.1 (9.8)	1.3 (0.7)	0.6	54.6 (3.9)	4.8 (0.8)	8.1
117	196.3 (8.8)	16.3 (7.0)	7.7	205.0 (15.6)	3.1 (1.6)	1.5	44.4 (0.5)	1.3 (0.7)	2.8
165	213.8 (11.4)	33.1 (3.9)	13.4	192.5 (12.0)	14.4 (7.9)	7.0	50.4 (2.4)	4.6 (0.4)	8.4
357	-	-	-	-	-	-	47.9 (2.8)	19.8 (13.5)	29.2

* Values for trophozoite (n=4) and cyst (n=4) populations are $x10^3$ cells/ml.

Figures in parenthesis are +/- one standard error.

was a delay prior to any large decrease in prey population (Fig.4.7). With Synechococcus there was no visible decline in population over the duration of the experiment, although microscopic observation of samples revealed many trophozoites containing numerous Synechococcus cells, often to the extent that they appeared bright green. Gross visual assessment of the control wells for the three prey species indicated no change in the cyanobacterial population during the experiment. Counts of Anabaena numbers in the control wells after 93h did not differ markedly from the initial population levels (Table 4.7).

The trophozoite and cyst populations at each sampling, and also the percentage of the amoebal population present as cysts, are listed in Table 4.7. With the two Anabaena spp. as the food source, cysts first appeared after 69h for the high amoebal inoculum and after 93h for the low amoebal inoculum. Both the size of the cyst population and the percentage encystment (%E) increased with subsequent samplings. After 165h %E was 28.6% in the presence of Anabaena A4 and 28.0% in the presence of A. variabilis at the low amoebal inoculum level, whilst values for the high amoebal inoculum were considerably less (13.4% and 7.0% respectively). Encystment also occurred in those amoebal populations fed Synechococcus (maximum of 29.2% after 357h for the high amoebal inoculum).

Although the initial populations of the three cyanobacterial species were standardized on the basis of optical density of cell suspensions, the biomass of food available to the acanthamoebae was not necessarily the same in each case. For this reason the overall extent of amoebal proliferation on the three prey species was not a

reliable indicator of the relative suitability of each as a ood source. A more satisfactory indicator was the amount of amoebal proliferation over periods of incubation during which the food source was present in abundance and not limiting amoebal growth. The initial cyanobacterial populations were intentionally high in relation to the numbers of amoebae present so as not to limit the predation rate, at least in the initial stages. The ratio of the number of Anabaena A4 cells to each trophozoite at the commencement of the experiment was 3 783:1 and 654:1 for the low and high amoebal inoculum levels, respectively, the corresponding values for A. variabilis being 2 482:1 and 429:1.

In those wells which received the low amoebal inoculum there were still considerable cyanobacterial populations remaining after 45h incubation ($16.9x10^6$ cells/ml for Anabaena A4 and $4.5x10^6$ cells/ml for A. variabilis -see Fig.4.7). During this period of the experiment the availability of Anabaena was therefore unlikely to have restricted amoebal proliferation, though it may be that by this stage A. variabilis is beginning to be limiting (40 cells to each amoeba present as opposed to 274 for Anabaena A4). Over the 45h period the Acanthamoeba population increased from $7.8x10^3$ cells/ml to $61.9x10^3$, $111.9x10^3$ and $21.7x10^3$ cells/ml in the presence of Anabaena A4, A. variabilis and S. leopoliensis respectively. These were increases of 7.9, 14.3 and 2.8-fold over the initial amoebal population. Each increase in amoebal population (i.e. each individual increase in cell number) was at the expense of 231 cyanobacterial cells for Anabaena A4, compared with 126 cells for A. variabilis.

Similarly, for wells with the high amoebal inoculum, amoebal

proliferation was unrestricted by the availability of food during the first 18h of incubation (after 18h there were 58 A. variabilis cells to each amoeba present, compared with 185 cells for Anabaena A4). Acanthamoeba numbers after 18h incubation were 100.6×10^3 cells/ml with Anabaena A4 as the food source, 137.5×10^3 cells/ml with A. variabilis and 39.4×10^3 cells/ml with S. leopoliensis. These were increases over the initial amoebal population of 2.2-fold with Anabaena A4 and 3.1-fold with A. variabilis. With Synechococcus as the food source the Acanthamoeba population decreased to 90% of the initial level. Each increase in amoebal population was at the expense of 194 cyanobacterial cells for Anabaena A4 and 122 cells for A. variabilis.

(3) The effect of pH on predation

The Anabaena A4 populations after 4d and 8d incubation for five pH values in the range 7.2-9.0 are given in Table 4.8, and the corresponding values for the A. castellanii population in Table 4.9. Amoebal and cyanobacterial populations after 4d incubation, over the same range of pH values, are plotted in Fig.4.8. The initial cyanobacterial population was 29.8×10^6 cells/ml, whilst that of the amoebae was 39.1×10^3 cells/ml. Over the range 7.2-9.0, reduction of the Anabaena A4 population by amoebal predation became progressively less as the alkalinity increased. After 4d the Anabaena population was reduced to 9.8×10^6 cells/ml (34.6% of the control population) at pH 7.2, and 18.0×10^6 cells/ml (60.2% of control) at pH 7.6 (Table 4.8, Fig.4.8). At the remaining three pH levels there was only a slight reduction in cyanobacterial numbers compared to the controls. These quantitative measurements were confirmed by visual assessment of the

Table 4.8. The effect of pH on the predation of Anabaena A4 by Acanthamoeba castellanii PB. Cyanobacterial numbers in experimental* (E) and control* (C) wells, and the experimental population as a percentage of the control, after 0, 4 and 8 days incubation at 20°C

pH	ANABAENA A4 POPULATION (x10^6 cells/ml)*						
	0d incubation	4d incubation			8d incubation		
	(n=18)	E (n=9)	C (n=9)	E as % of C	E (n=9)	C (n=9)	E as % of C
7.2	29.8 (2.0)	9.8 (1.2)	28.3 (1.7)	34.6	1.1 (0.2)	32.4 (2.2)	3.4
7.6	29.8 (2.0)	18.0 (2.4)	29.9 (4.7)	60.2	15.9 (2.0)	25.7 (3.8)	61.9
8.0	29.8 (2.0)	23.3 (2.9)	26.3 (4.2)	88.6	17.3 (3.1)	20.8 (4.3)	83.2
8.6	29.8 (2.0)	25.4 (4.3)	26.1 (7.8)	97.3	21.1 (3.7)	22.6 (3.5)	93.4
9.0	29.8 (2.0)	24.5 (4.4)	27.9 (4.6)	87.8	17.8 (2.2)	25.0 (5.1)	71.2

*Both control and experimental wells had an initial cyanobacterial population of 29.8x10^6 cells/ml. Experimental wells additionally contained an initial amoebal population of 39.1x10^3 cells/ml. Figures in parenthesis are plus/minus 95% confidence intervals.

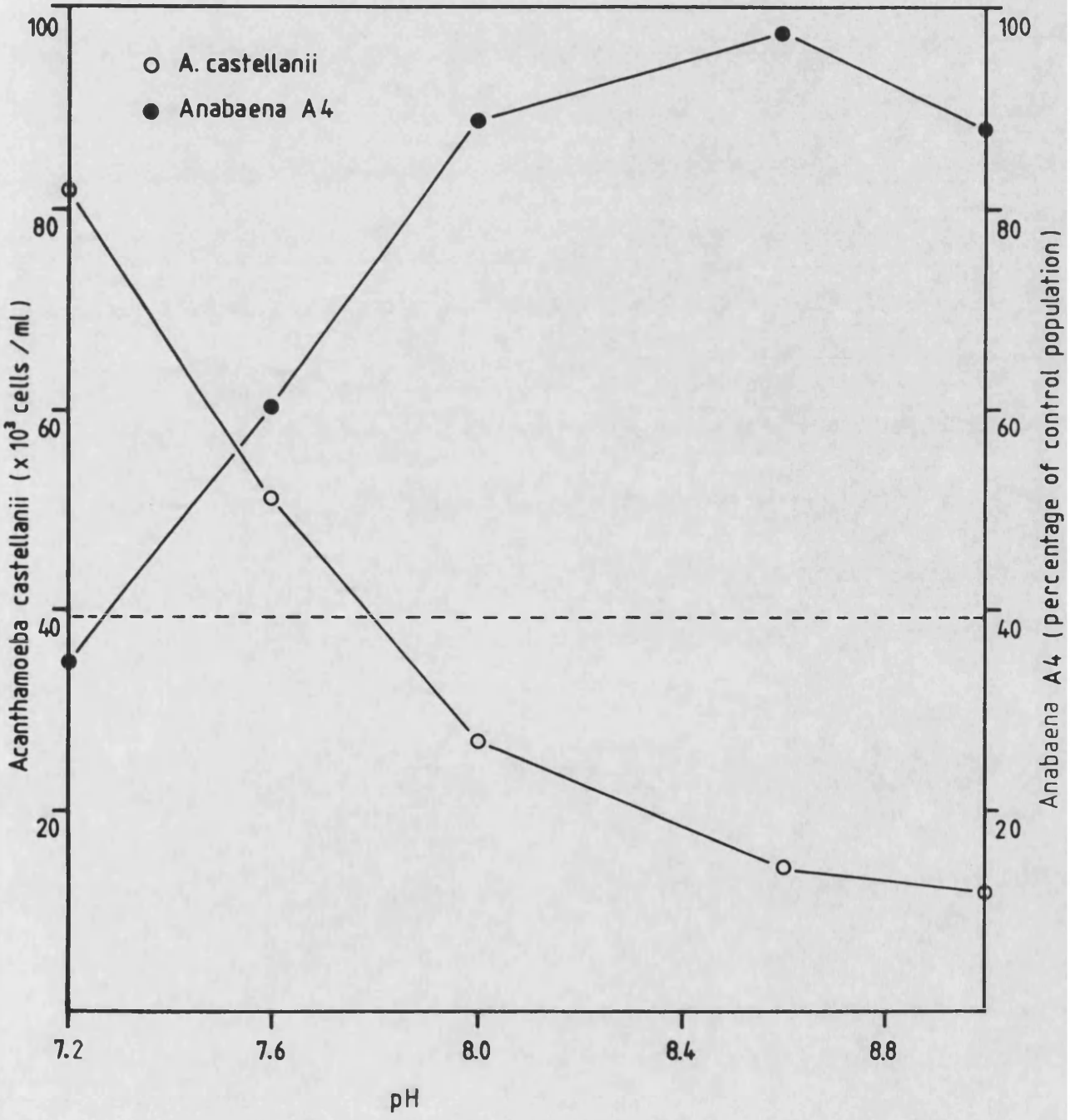
Table 4.9. The effect of pH on the predation of Anabaena A4 by Acanthamoeba castellanii PB: Amoebal populations after 0, 4 and 8 days incubation at 20°C.

pH	Acanthamoeba castellanii population* ($x10^3$ cells/ml)		
	0 Days	4 Days	8 Days
7.2	39.1 (2.5)	81.8 (11.6)	66.3 (10.8)
7.6	39.1 (2.5)	51.1 (6.4)	37.4 (6.4)
8.0	39.1 (2.5)	26.8 (7.7)	14.0 (7.5)
8.6	39.1 (2.5)	14.4 (2.3)	9.3 (3.3)
9.0	39.1 (2.5)	11.9 (3.3)	4.0 (1.8)

* All values are the mean of six counts. Figures in parenthesis are plus/minus 95% confidence intervals.

Fig.4.8. The effect of pH on the predation of Anabaena A4 by Acanthamoeba castellanii PB. Amoebal and cyanobacterial populations after four days incubation at 20°C.

(Cyanobacterial numbers are expressed as a percentage of the control population. The dotted line indicates the amoebal population at time of inoculation - 39.1×10^3 cells/ml).



wells, which revealed marked clearing at pH 7.2, limited clearing at pH 7.6 and no detectable clearance at the higher pH values. The changes in amoebal population over the same period (4d) were closely correlated with the extent of reduction of the Anabaena population. Acanthamoeba numbers increased over 4d to $81.8x10^3$ cells/ml at pH 7.2 and $51.1x10^3$ cells/ml at pH 7.6 (Fig.4.8), increases over the initial population of 109% and 31% respectively. By contrast, numbers declined to 69%, 37% and 31% of their initial level at pH 8.0, 8.6 and 9.0, respectively.

After 8d incubation there was a further marked reduction in the Anabaena population to $1.1x10^6$ cells/ml (3.4% of control population) at pH 7.2, but at the remaining pH levels there were no significant decreases in cell number when expressed as a percentage of the control population. Acanthamoeba numbers declined at all five pH values in relation to their level after 4d (Table 4.9).

There was no detectable clearance of the Anabaena population after either 4d or 8d incubation for those wells with pH values in the range 4.0-7.0. After 8d the Anabaena population at pH 7.0 was $15.9x10^6$ cells/ml, a level slightly greater than that of the control ($15.5x10^6$ cells/ml). With this exception, no counts were made of the predator and prey populations for these pH values. Microscopic observation of samples showed many amoebae to be dead, the remainder small and apparently starving.

Discussion

(1) Ingestion/predation of cyanobacteria in mixed cultures

For those experiments in which percentage ingestion was recorded, the initial Anabaena A4 population was high in relation to the numbers of amoebae present. This was intentional so that the rate of ingestion of cyanobacteria by amoebae would not be limited through lack of an available food source, at least over the initial stages of incubation. In the experiment comparing ingestion in the Allen's and PGY treatments there were initially 380 Anabaena cells for each amoeba present, whilst in that comparing ingestion at different temperatures there were originally 553 cyanobacterial cells for each amoebal cell.

Comparison of ingestion in Allen's and PGY treatments

The disparity in the pattern of ingestion between the Allen's and PGY treatments over the first few hours of incubation might result from one of two causes. Firstly, centrifugation of the amoebae prior to suspension in Allen's medium may have caused physical disturbance or damage to the cells which affected their ability to phagocytose Anabaena cells. However, microscopic observation of samples after centrifugation gave no indication of damage to the amoebae. The possibility of permanent damage was also contradicted by the observation that percentage ingestion in the Allen's treatment eventually reached the same high value as in the PGY treatment. Rounding-up of amoebae during centrifugation, involving the withdrawal of pseudopodia, would reduce the surface area over which phagocytosis could operate and in consequence might limit the ingestion rate of cyanobacteria. However, the interval between the resuspension of

amoebae in Allen's medium and their inoculation into Repli dish wells containing the cyanobacterial prey (45min) was sufficient for the amoebae to recover their normal cell shape prior to the commencement of the experiment (this was verified by light microscopy). A second, more probable cause of the disparity between treatments relates to the differences in the composition of the medium surrounding the amoebae in the two treatments. In the PGY treatment the amoebae were in effect still being cultured in half strength PGY medium (see materials and methods), whereas in the Allen's treatment the amoebae were surrounded by Allen's medium alone. As a consequence the medium in the PGY treatment would have been more suitable for the maintenance of amoebae than that in the Allen's treatment (in terms both of ionic strength/composition and pH) so promoting greater phagocytic uptake of cyanobacterial cells.

An interesting aspect of the PGY treatment was that the acanthamoebae were effectively presented with a choice of food, that of PGY medium or Anabaena cells. Yet despite this choice the uptake of cyanobacteria was more rapid than in the Allen's treatment, where no alternative food was available. The high value for percentage ingestion in the PGY treatment (22.5%) after only 15min incubation showed that the amoebae can very rapidly switch their mode of nutrition from pinocytosis (in this case uptake of solutes from PGY medium) to one in which phagocytosis is predominant (uptake of cyanobacterial cells).

The progressive decrease in percentage ingestion for both treatments during the latter stages of incubation was probably due to the prey becoming an increasingly limiting resource (amoebae did not

encounter cyanobacteria with sufficient frequency for the high level of ingestion to be maintained), rather than to any decline in the ability of the amoebal cells to phagocytose Anabaena.

The more rapid decrease in cyanobacterial population in the PGY treatment compared with the Allen's treatment corresponded well with the greater rate of ingestion of Anabaena cells by amoebae in this treatment, and also with the observations that the number of ingested cells per amoeba was greatest in the PGY treatment. These differences were not however reflected in the amoebal populations for the two treatments, which were very similar over the first 32h of incubation. The greater increase in amoebal population between 32h and 77h in the PGY treatment (Table 4.2) may indicate that the amoebae in this treatment were utilizing the solutes in the PGY medium to compensate for the increasing scarcity of cyanobacterial prey.

Effect of temperature

These experiments have shown that incubation temperature is a major determinant of the rate and extent of uptake of Anabaena cells by Acanthamoeba castellanii. Moreover, this effect becomes more critical as the temperature is reduced. For example, after 3h incubation at 30°C the value for percentage ingestion was 94%. The difference between this and the corresponding value for 20°C, 91.5%, was only 2.5%, yet the drop in percentage ingestion between 20°C and 10°C was 20%, and that between 10°C and 4°C, an interval of only six degrees, was 66%. These effects of temperature on endocytosis were in general agreement with the results of Bowers (1977), who found that both phagocytosis and pinocytosis in A. castellanii ceased below 5°C.

Phagocytosis (of latex beads) was low and variable from 10-15°C (less than 10% of the rate of uptake at 29°C). Above 16°C the rate of uptake was a linear function of temperature. Temperature may exert this strong effect on ingestion by its influence on membrane fluidity. Evidence suggests that fluidity is of crucial importance in membrane fusions (including those involved in phagocytosis) (Poste & Allison, 1973). However, most plasma membrane fatty acids in Acanthamoeba have a transition temperature below 0°C (Korn, 1963). Bowers (1977) has suggested that limitation of ATP is a more likely contributing factor to the decrease of endocytosis with lowered temperatures. In a more general sense temperature may restrict the mobility of amoebal trophozoites and so reduce the probability of contact with the prey species.

Temperature, as an inevitable consequence of its effect on ingestion rate, largely determined the rate and extent of cyanobacterial decline and amoebal proliferation in the experimental wells. With few exceptions, increased incubation temperature led to greater ingestion of prey, more rapid decline in the cyanobacterial population and greater amoebal proliferation. The differences in the development of the Acanthamoeba population at 20°C and 30°C were surprising in view of the similarity in the rate and extent of decline of the Anabaena population at the two temperatures. The supply of food was constant at both temperatures, yet it supported considerably greater proliferation of amoebae at 20°C than at 30°C (Table 4.6, Fig.4.4). The amoebae were no larger at 30°C than at 20°C, and so the difference in amoebal populations was one of biomass as well as numbers. These observations suggest that conditions for amoebal

reproduction were nearer the optimum at 20°C than at 30°C. At the higher temperature a greater proportion of the energy contained in the food may have been lost through elevated amoebal respiration, resulting in a smaller increase in amoebal population than at 20°C.

Similar results to those of the present study have been reported by Yamamoto (1981) and Huang & Wu (1982). The former author investigated the predation of Nuclearia on Anacystis nidulans in liquid culture. At 4°C there was no growth of amoebae, whereas at 28°C there was extensive amoebal proliferation with a corresponding decrease in cyanobacterial population. Huang & Wu (1982) found that both a Nuclearia sp. and an Anabaena sp. virtually eliminated Anabaena populations after 8d incubation in liquid culture. Exhaustion of cyanobacteria was followed by encystment of amoebae. Predation by these amoebae was "normal" between 20°C and 40°C.

The occurrence of encysted amoebae in those treatments in which predation of the Anabaena was most complete, and at a stage of incubation in which the cyanobacterium was severely limiting, suggests that encystment was initiated in response to a shortage of food. The ratio of Anabaena cells to amoebae at the time cysts were first recorded was 9:1 at 30°C and less than 1:1 at 20°C. Once the prey was exhausted the only alternative to encystment was gradual starvation followed by death.

(2) Comparison of the predation of three cyanobacterial species

This experiment was conducted at 20°C on the basis of evidence, obtained in a previous part of this study, that amoebal proliferation

upon cyanobacteria in Repli dish cultures was maximal at this temperature (see p 96).

Under the conditions imposed by the experiment the three cyanobacterial species fell into two distinct groups with respect to their suitability as a food source for Acanthamoeba castellanii strain PB. The two Anabaena species were readily predated and supported large increases in the amoebal population, whereas the Synechococcus was consumed to a much smaller extent and only supported a modest rise in numbers of Acanthamoeba. The inability of the amoebae to effectively utilize Synechococcus was surprising in view of the ease with which it was predated in lawn cultures and also in large volume (100ml) batch cultures (see Sections one and three, respectively).

The occurrence of a delay in wells containing the low amoebal inoculum, but not the high amoebal inoculum, prior to any appreciable decrease in the cyanobacterial population, was directly attributable to the difference in the size of the initial predator populations. With the high amoebal inoculum the great grazing pressure of the initial Acanthamoeba population ($45x10^3$ cells/ml) led to an immediate reduction in the Anabaena population. However, with the low amoebal inoculum ($7.8x10^3$ cells/ml) it was first necessary for Acanthamoeba numbers to build up before any impact could be made on the cyanobacterial population.

With Anabaena as a food source the onset of amoebal encystment was closely related to the exhaustion of cyanobacterial prey. Confirming this was the observation that encystment first occurred in those wells inoculated with the high amoebal density, and which in consequence had the most rapid depletion in cyanobacterial numbers.

There was no obvious reason for the differences in the values of %E between the two levels of amoebal inoculum. With Synechococcus as a food source, encystment of amoebae occurred despite the continued availability of cyanobacterial prey.

Although both Anabaena spp. supported similar increases in amoebal population (Fig.4.6), this did not necessarily mean, for the reasons outlined earlier (see results section), that the two species were equally suitable as food sources for Acanthamoeba. Indeed, the comparisons of amoebal proliferation during periods of incubation in which there was a plentiful supply of cyanobacterial prey indicate the contrary. For example, with the low amoebal inoculum the increase in amoebal population over the first 45h of incubation was almost twice as great with Anabaena variabilis as food (14.3-fold increase) as with Anabaena A4 (7.9-fold increase). A similar disparity occurred over the first 18h of incubation for the high amoebal inoculum, with A. variabilis supporting a 3.1-fold increase in amoebal numbers compared with a 2.2-fold increase for Anabaena A4. These observations provide strong evidence that, in terms of its ability to support amoebal proliferation, A. variabilis is a better food source for A. castellanii PB than Anabaena A4. The reason for this may lie in differences in the nutritional value or efficiency of assimilation of the two species by Acanthamoeba.

Despite the greater suitability of A. variabilis as a food source, the differences in the number of cells of each Anabaena species required to support each individual increase in cell number of the amoebal population (the values of which were consistent for both amoebal inoculum levels) can be explained largely by the size

disparity between the two prey species. The A. variabilis cells measured 5.5 +/- 0.49* µm by 4.3 +/- 0.27* µm (n=25) and those of Anabaena A4 4.0 +/- 0.3* µm by 3.4 +/- 0.1* µm (n=25). The smaller dimensions of the latter dictated that, even assuming both Anabaena spp. had equal nutritional value, a greater number of Anabaena A4 cells would have to be consumed in order to support a given increase in amoebal population.

*95% confidence limits

(3) The effect of pH on predation

Although data were only obtained for pH values in the range 7.2-9.0, certain trends were clearly discernable. Whilst predation of the Anabaena population occurred at those pH values closest to neutrality (pH 7.2, pH 7.6), under more alkaline conditions it was effectively prevented. The increase in numbers of Acanthamoeba at the lower pH values in this range (Table 4.9) was a direct response to the consumption of Anabaena. At higher pH values the amoebae were unable to utilize the cyanobacterial food source, leading to starvation and a subsequent decrease in population. This decrease was more marked the greater the alkalinity, suggesting that conditions were increasingly unsuitable for the maintenance of amoebae. The decline in Anabaena populations in the control wells was not unexpected considering that the cyanobacteria were suspended in a non-nutrient solution, and incubated in the dark, conditions that precluded both growth and maintenance of cells.

The lack of predation by amoebae suspended in citrate phosphate

buffer (pH range 4-7) was most likely caused by toxicity of the buffer to the amoebae rather than to an inability of the amoebae to predate the cyanobacteria at any of the six pH levels tested. Predation would at least be expected at those pH levels approaching neutrality, especially in view of the large decreases in Anabaena recorded for Tris buffer at pH 7.2. Indeed, predation by A. castellanii under slightly acidic conditions has been reported (Ho & Alexander, 1974). These authors compared the proliferation of several amoebal species upon both cyanobacteria and eukaryotic algae in organic salts medium prepared in 0.2M phosphate buffer (pH 6.0) and 0.2M Tris buffer (pH 8.0). Hartmannella castellanii (= A. castellanii) proliferated at pH 6.0, but not at pH 8.0, whereas for Amoeba discoides the reverse was true. Both species grew at neutrality. Of the other authors who have investigated the effect of pH on the predation of cyanobacteria by protozoa, Yamamoto (1981) recorded the mean doubling time (at 28°C) of an isolate of Nuclearia over the pH range 5-10 when fed three cyanobacterial species. The optimum pH for predation was 8.0 with Microcystis aeruginosa and Anacystis nidulans as food, and 6.0 with Anabaena cylindrica. Huang & Wu (1982) found that predation by an Amoeba sp. and a Nuclearia sp. upon several filamentous cyanobacteria was "normal" at pH 5-9.

SECTION THREE

PREDATION IN ILLUMINATED BATCH CULTURES

Introduction

In this section further detailed studies are made of the predation of Anabaena A4, and to a lesser degree Synechococcus leopoliensis 1405/1, by Acanthamoeba castellanii strain PB. The relatively large volumes used in these experiments (100ml in 250ml Erlenmeyer flasks) conferred two important advantages over the Repli dish cultures employed in the previous section. Firstly, they allowed repeated sampling from individual flasks, as opposed to the destructive sampling regimes which were needed for Repli dish cultures. It was therefore possible to monitor temporal changes in individual populations of both amoebae and cyanobacteria. Secondly, a larger volume reduced the problem of gradual evaporative loss, so enabling incubation of cultures to continue for a longer period.

Aside from the above factors, the most important feature of the experiments in this section was that mixtures of predator and prey were incubated in the light in the normal cyanobacterial growth medium. These conditions provided an opportunity for the photosynthesis (and consequent proliferation) of the cyanobacterial food source. As a consequence, amoebal predators were presented with a potentially expanding food reservoir, rather than a food pool of fixed size such as that seen in the previous section.

Experiments were designed to compare the predation of the two cyanobacterial species (Anabaena A4 and Synechococcus leopoliensis)

under similar conditions, and also to investigate the effect of incubation temperature, and varying the relative proportions of amoebae and cyanobacteria, on the subsequent changes in predator and prey populations.

Materials and Methods

Establishment of mixed cultures of predator and prey

Amoebal and cyanobacterial inocula were added to 90ml sterile Allen's medium in 250ml cotton-wool plugged Erlenmeyer flasks. Cyanobacteria were inoculated directly from established batch cultures (approx. 21d). The density of cyanobacterial inocula was monitored by measuring the optical density of whole cell suspensions at 680nm. Amoebal inocula were prepared by centrifuging (10min, 500g) samples from a 2d batch culture. The trophozoites were resuspended in Allen's medium, which was then added to the experimental flasks. Control flasks received a volume of sterile Allen's medium equivalent to the size of the amoebal inoculum. Flasks were incubated statically, but were swirled daily to resuspend the predator and prey populations. Precise details of the inoculum sizes and incubation temperature for each experiment are given below. In all cases flasks were incubated under constant illumination (photosynthetically active radiation of 13 microeinsteins $m^{-2}s^{-1}$).

(A) Comparison of the predation of Anabaena A4 and Synechococcus leopoliensis 1405/1

Four flasks (two experimental, two control) were established for each of the two cyanobacterial species. Cyanobacterial inocula

consisted of 2ml and 3ml of Synechococcus and Anabaena cultures respectively. Both sets of flasks were inoculated with 5ml of a 2d batch culture of Acanthamoeba, resuspended in a similar volume of Allen's medium. The final volume of medium in each flask was adjusted to 100ml with sterile Allen's medium.

Samples were removed immediately after inoculation of amoebae and after 42, 85, 133, 213, 277, 352, 477 and 1049h incubation. Two samples (0.5ml) were taken from each flask for counts of the amoebal population.

(B) The effect of temperature on predation

Six flasks (four experimental, two control) were established for each of three temperatures- 15°C, 22.5°C and 30°C. Each contained 90ml sterile Allen's medium which was inoculated with 3ml of a suspension of Anabaena cells. Experimental flasks were inoculated with 5ml of a 2d batch culture of Acanthamoeba resuspended in 5ml Allen's medium. Control flasks received 5ml Allen's medium. The final volume in each flask was 98ml.

Samples were removed for quantification of predator and prey populations after 0, 20, 44, 70, 92, 115 and 145h incubation (one sample per flask for amoebal counts).

(C) The effect of varying amoebal inoculum size

Each of eleven flasks containing 90ml sterile Allen's medium was inoculated with 6ml Anabaena A4 culture. Two flasks were inoculated with each of the four densities of amoebal inoculum. These inocula consisted of 1.0, 2.5, 5.0 and 10.0ml of a 2d batch culture of

Acanthamoeba resuspended in 4ml Allen's medium. The three remaining flasks, to which were added 4ml Allen's medium, acted as controls.The final volume in each flask was 100ml.

Flasks were incubated at 15°C. Samples were removed after 0, 18, 47, 66, 91, 134, 165, 205, 256, 325 and 449h incubation.

(D) The effect of varying cyanobacterial inoculum size

Flasks were set up containing four different levels of cyanobacterial inoculum, represented by 3, 6, 9 and 12ml of Anabaena suspension. Five flasks were established for each inoculum level, of which three were inoculated with amoebae (5ml of a 2d batch culture resuspended in 5ml Allen's medium) and the remaining two acting as controls. The volume of medium in each flask was adjusted to 100ml with sterile Allen's medium.

Flasks were incubated at 20°C. Samples were removed after 0, 10, 25, 44, 70, 92, 122 and 169h incubation (one sample per flask for amoebal counts).

Quantification of predator and prey populations

Samples were removed aseptically immediately after inoculation of flasks and at intervals thereafter (see above). The flask contents were mixed thoroughly by swirling before withdrawal of samples.

(1) Cyanobacteria

Chlorophyll a concentration was used as a measure of the cyanobacterial population in the flasks. Chlorophyll a was extracted from the cyanobacterial cells using a hot methanol treatment.

Calibration: The relationship between the absorbance of chlorophyll a

extracts and cyanobacterial cell density was determined for both the cyanobacterial species used in these experiments, Anabaena A4 and Synechococcus leopoliensis 1405/1. For each species a dilution series was prepared in centrifuge tubes (15ml) from a 5ml sample from a batch culture (14d) of the cyanobacterium (two-fold dilution at each step, sterile Allen's medium used as the diluent). The volume of all diluted cell suspensions was 5ml. cells were pelletted by centrifugation at 2000g for 25min. The supernatant from each tube was discarded and the cells resuspended in 5ml 95% (v/v) methanol, a Pasteur pipette being used to disperse large clumps of cells. Chlorophyll a was extracted by placing the tubes in a heated water bath at 70°C for 10min. Samples were recentrifuged and the supernatant for each dilution transferred to a 5ml volumetric flask. Each extract was then adjusted to 5ml with 95% (v/v) methanol.

To construct curves relating the absorbance of chlorophyll a to cyanobacterial density the absorbance of extracts was read at 665nm in 1cm path length polystyrene cuvettes (4.5ml capacity) against a methanol blank using a Pye-Unicam SP6-550 UV/VIS spectrophotometer.

To determine the position and height of absorbtion peaks, chlorophyll extracts from the two cyanobacterial species were wavelength scanned between 555 and 730nm at 0.3nm intervals, against a methanol blank, using an LKB Ultrospec 4050.

Sample treatment: One sample (2ml) from each of the experimental flasks (containing mixed populations of predator and prey) and the control flasks (containing only cyanobacteria) was taken through the extraction sequence. Following extraction in methanol, supernatants

were transferred to 2ml volumetric flasks and the volume adjusted to 2ml with 95% (v/v) methanol. The absorbance of extracts was read at 665nm in 1cm path length polystyrene micro-cuvettes (2.9ml capacity) against a methanol blank.

The chlorophyll a concentration was calculated using the formula of Talling & Driver (1963).

$$C_a = 13.9 \times D_{665}$$

Where C_a = Chlorophyll a concentration (mg/l)

D_{665} = Absorbance at 665nm in 95% methanol.

(2) Amoebae

Trophozoite and cyst numbers were determined by haemocytometer counts. One or two samples (0.5ml), depending on the experiment, from each experimental flask were transferred to Repli dish wells and fixed by adding an equal volume of 3% (v/v) glutaraldehyde in distilled water. Dishes were sealed with tape and stored at room temperature pending counts. The well contents were mixed (to evenly resuspend cells) and the volume adjusted to 1ml with distilled water (to compensate for any evaporative loss during storage) prior to counting. Two counts were made for each sample. Samples containing dense populations of amoebae were diluted with tap water to facilitate counting. A volume of fluid equal to that removed from the experimental flasks was removed from the controls and discarded, thereby standardizing the volume reduction at each sampling.

Results and Discussion

Chlorophyll Extraction

Plots of the absorbance at 665nm of extracts from serially diluted cyanobacterial cultures gave a straight-line relationship for both Anabaena and Synechococcus provided that absorbance values were in the range 0.01 to 1.0. A representative plot for Synechococcus is given in Fig.5.1. Outside this range, linearity was lost for both low and high absorbance values. From this it is evident that, within the stated range, absorbance by chlorophyll a is directly proportional to the cyanobacterial population of the sample, and as such is a reliable measure of the size of that population.

Wavelength scans of methanol extracts from the two cyanobacterial species over the range 555-730nm gave absorbance peaks at 664.7nm for Anabaena A4 and at 664.5nm for Synechococcus leopoliensis 1405/1 (Fig.5.2). These findings confirm the validity of using a wavelength of 665nm for the measurement of Anabaena and Synechococcus populations.

(A) Comparison of the predation of Anabaena A4 and Synechococcus leopoliensis 1405/1

Results

The effect of predation by Acanthamoeba castellanii PB on the populations of Anabaena and Synechococcus, measured in terms of the reduction in chlorophyll a levels, is shown in Fig.5.3 together with the development of the amoebal cyst and trophozoite populations. Table 5.1 gives the trophozoite and cyst numbers, total amoebal count and

Fig.5.1. Absorbance at 665nm of chlorophyll a extracts from serially diluted Synechococcus leopoliensis suspensions.

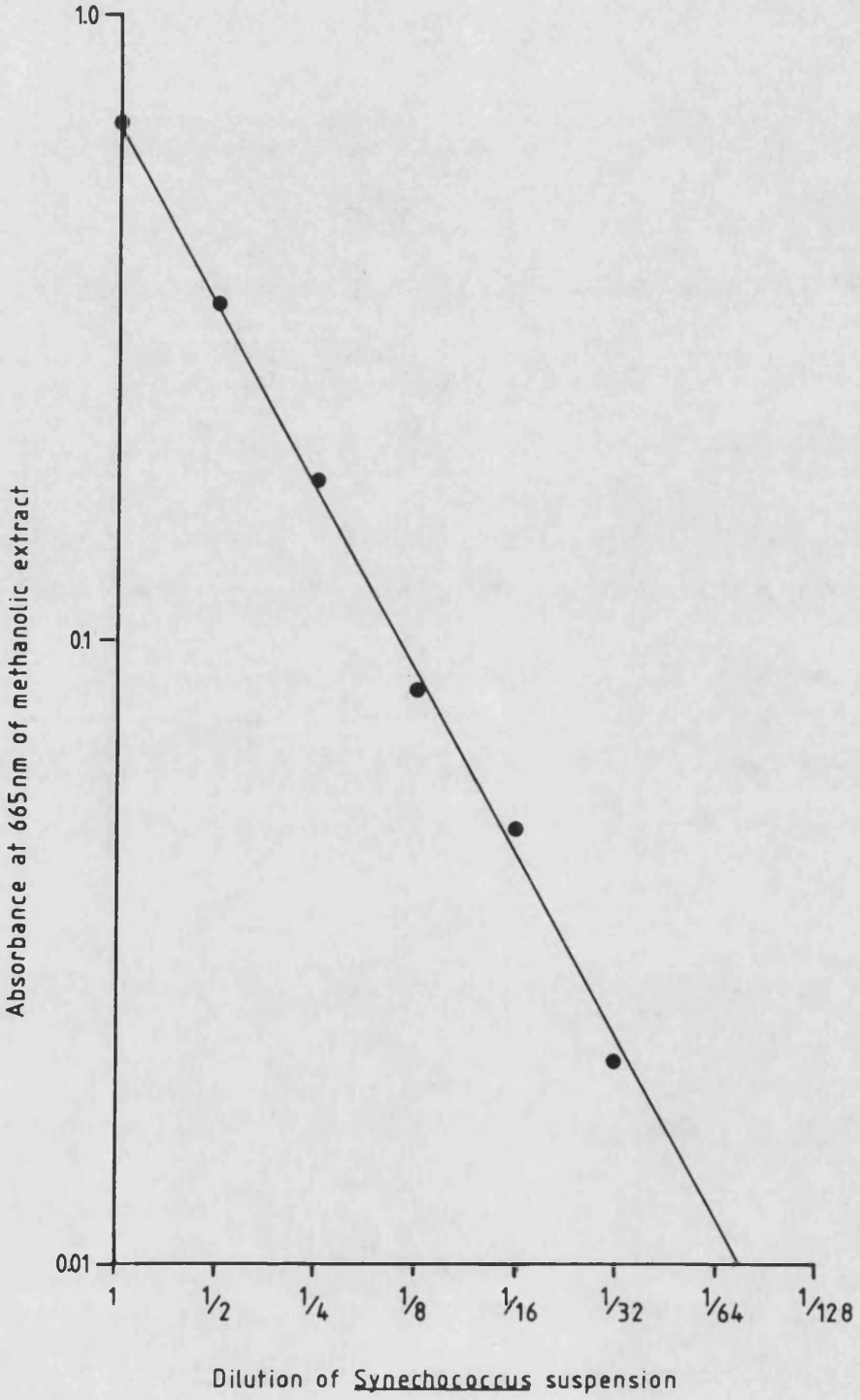
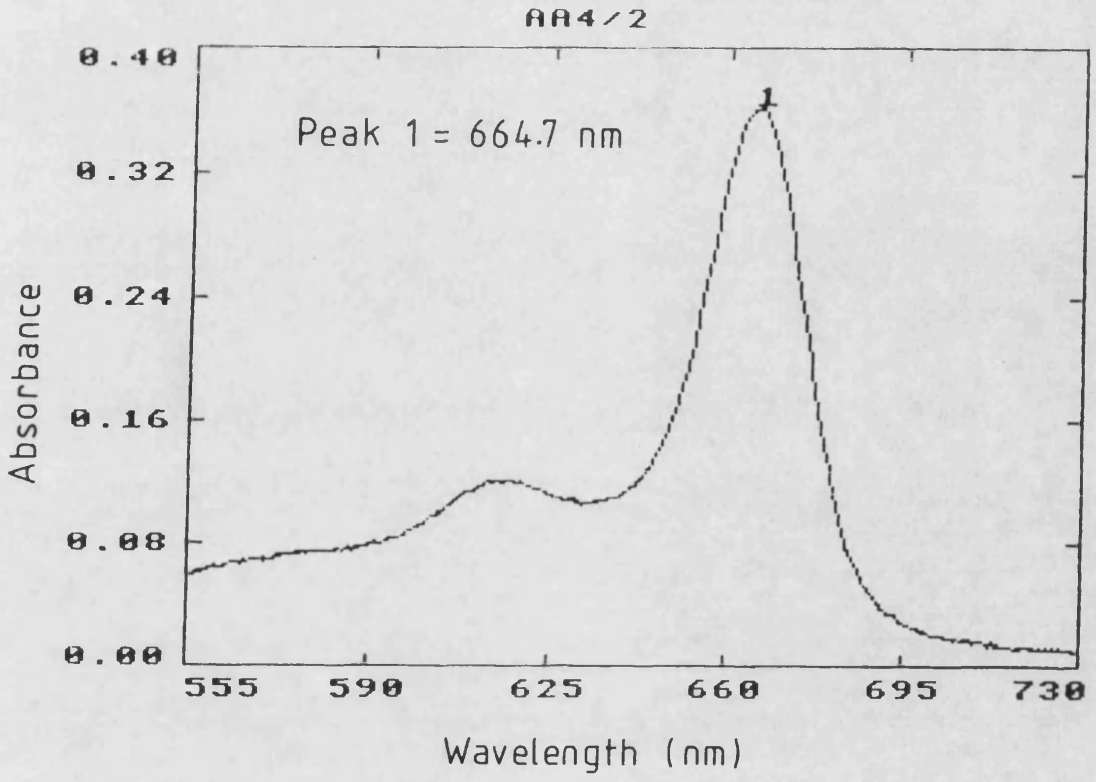


Fig.5.2. Wavelength scans of methanol extracts from cell suspensions of Anabaena A4 and Synechococcus leopoliensis.

(A) Anabaena A4



(B) Synechococcus leopoliensis 1405/1

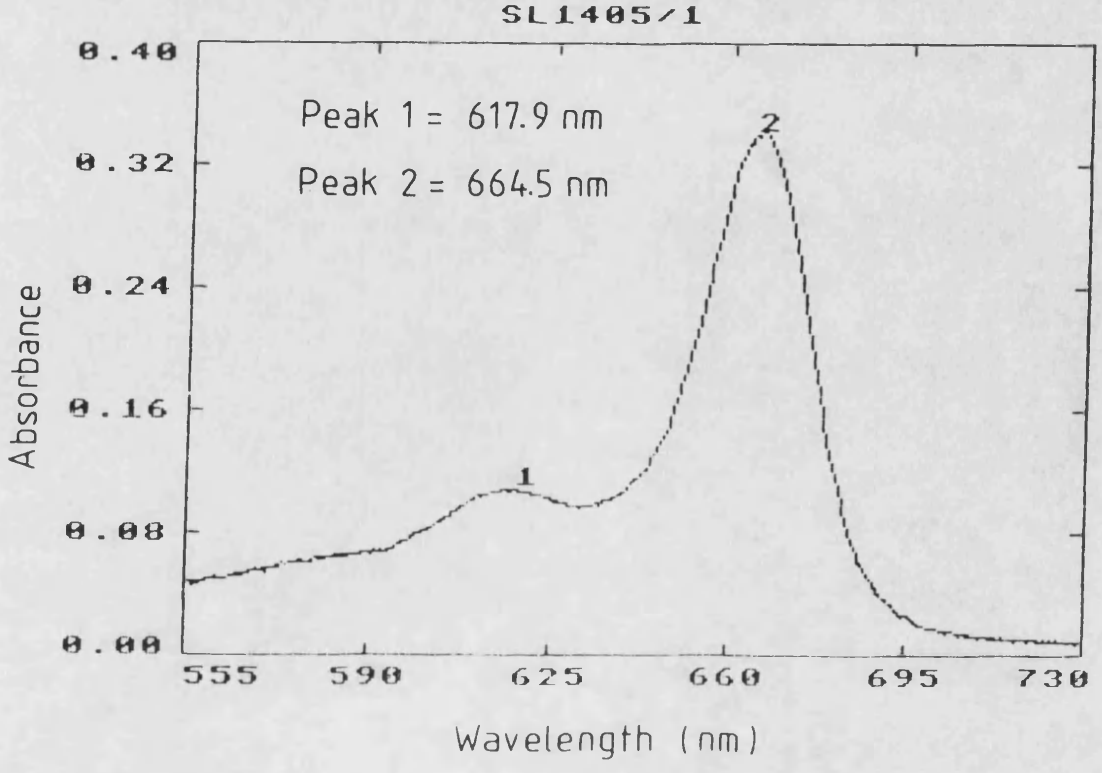


Fig.5.3. Predation of Anabaena A4 and Synechococcus leopoliensis 1405/1 by Acanthamoeba castellanii PB in illuminated batch cultures at 15°C. Changes in amoebal and cyanobacterial populations with incubation time.

○ Anabaena population (control, n=2)

● Anabaena population (experimental, n=2)

□ Trophozoites (n=8)

■ Cysts (n=8)

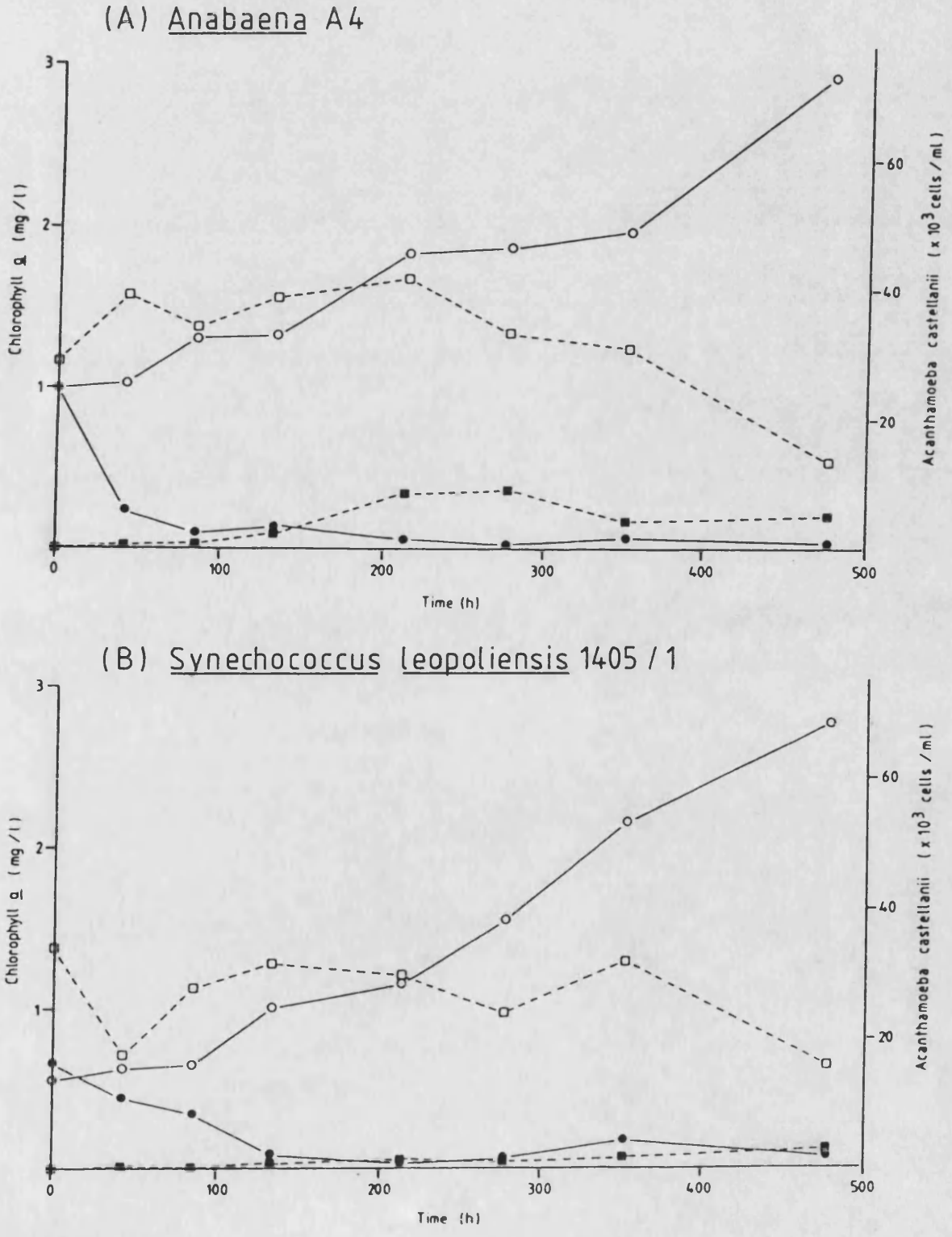


Table 5.1. Changes in A. castellanii PB population (trophozoites (T), cysts (C) and total amoebal count (TAC)), and percentage of the amoebal population present as cysts (%E), during incubation at 15°C with either Anabaena A4 or Synechococcus leopoliensis as the food source.

Time (h)	ACANTHAMOEBA CASTELLANII POPULATION ($x10^3$ cells/ml)*											
	(Anabaena as food)						(Synechococcus as food)					
	T	C		TAC		%E	T	C		TAC		%E
0	29.2 (2.0)	0	-	29.2	(2.0)	0	34.6	(1.7)	0	-	34.6	(1.7)	0
42	39.5 (1.8)	0.7	(0.4)	40.2	(1.6)	1.7	17.9	(1.7)	0.6	(0.3)	18.5	(1.8)	3.2
85	34.6 (2.5)	1.0	(0.4)	35.6	(2.6)	2.8	28.3	(2.0)	0.3	(0.3)	28.6	(2.0)	1.0
133	39.0 (2.7)	2.4	(0.6)	41.4	(3.3)	5.8	31.8	(2.3)	0.8	(0.6)	32.6	(2.4)	2.5
213	42.0 (2.9)	8.6	(1.0)	50.6	(2.4)	17.0	30.0	(2.4)	1.4	(0.5)	31.4	(1.9)	4.5
277	33.5 (2.9)	9.2	(1.2)	42.7	(3.5)	21.5	24.2	(2.5)	1.1	(0.8)	25.3	(2.4)	4.3
352	31.1 (1.6)	4.3	(0.5)	35.4	(1.4)	12.1	32.2	(1.2)	1.7	(0.3)	33.9	(1.4)	5.0
477	13.6 (2.2)	5.1	(0.6)	18.7	(2.4)	27.3	15.8	(0.8)	2.8	(1.0)	18.6	(1.1)	15.1
1049	0 -	8.3	(1.6)	8.3	(1.6)	100.0	24.4	(1.6)	3.6	(1.4)	28.0	(1.2)	12.9

* All values are the mean of eight counts. Figures in parenthesis are +/- one standard error.

percentage of the amoebal population present as cysts for each sampling. The Acanthamoeba inoculum (Approximately $30x10^3$ cells/ml) was the same for both cyanobacterial species.

The decline in cyanobacterial population over the initial stages of incubation was more rapid for Anabaena than Synechococcus. This was despite the fact that the cyanobacterial population, in terms of chlorophyll a content, was initially considerably higher for Anabaena (0.99 mg/l) than for Synechococcus (0.67 mg/l). According to chlorophyll a levels, after 85h incubation the Anabaena population had been reduced by 90%, and that of Synechococcus by 48%. Over the same period the populations in control flasks increased by 31% and 17% for Anabaena and Synechococcus, respectively. The cyanobacterial populations further decreased with continued incubation, both species declining, after 213h, to 6% of their initial level. These values were equivalent to 3.4% and 3.7% of the control population for Anabaena and Synechococcus respectively. Microscopic observation of samples after 277h revealed that the Anabaena population in the experimental flasks had been totally eliminated, despite the small positive values obtained from chlorophyll extraction. By contrast, the Synechococcus flasks still contained small numbers of cyanobacterial cells, a situation which continued until 477h incubation. Subsequently there was a recovery in cell numbers, so that after 1049h (44d) the Synechococcus population had increased to almost six times its initial level. Representative control and experimental flasks for the two cyanobacterial species after 7 and 44d incubation are shown in Fig.5.4. The contrast between the elimination of the Anabaena population and the regrowth of the Synechococcus population after 44d

Fig.5.4. Predation of Anabaena A4 and Synechococcus leopoliensis 1405/1 by Acanthamoeba castellanii PB in illuminated batch cultures (15°C). Control flasks (minus amoebae) on left, experimental flasks (initial amoebal population of $30x10^3$ cells/ml) on right.

(A) Anabaena A4

7 days incubation

44 days incubation

Fig.5.4. (Continued).

(B) Synechococcus leopoliensis 1405/1.

7 days incubation

44 days incubation

can clearly be seen.

Predation of cyanobacteria was accompanied by changes in the amoebal population (Table 5.1, Fig.5.3). With Anabaena as a food source the total amoebal count (TAC - trophozoites and cysts combined) increased from $29x10^3$ to $51x10^3$ cells/ml over the first 213h of incubation. Thereafter the TAC gradually declined to $18.7x10^3$ cells/ml after 477h and $8.3x10^3$ cells/ml after 1049h. Trophozoite numbers fluctuated between $30x10^3$ and $40x10^3$cells/ml over the first 200h incubation, then slowly decreased. By contrast, cyst numbers increased with incubation time. Encystment was first recorded after 42h, reaching a peak of $9.2x10^3$ cells/ml after 277h. The proportion of the Acanthamoeba population present as cysts also increased during incubation, reaching a maximum of 100% after 1049h (Table 5.1). With Synechococcus as the food source there was no increase in TAC, amoebal numbers in fact falling over the first 42h of incubation. Thereafter both the TAC and trophozoite numbers fluctuated between $15x10^3$ and $34x10^3$ cells/ml. Cyst numbers reached a peak of $3.6x10^3$ cells/ml after 1049h. Percentage encystment was much lower than for Anabaena, with a maximum value of 15.1% after 477h.

Discussion

The results have demonstrated that, under the particular conditions of the experiment, Acanthamoeba castellanii PB was effective in controlling the populations of both cyanobacterial species, at least in the first instance. Suspension of cyanobacterial cells in their normal growth medium, and incubation in the light, permitted the proliferation of Anabaena and Synechococcus populations

during incubation. Even so predation was still effective. The rate of ingestion of cyanobacteria by amoebae was more than sufficient to offset the rate of increase in cyanobacterial population size, thereby resulting in an overall decrease in cyanobacterial numbers.

The observed differences in the predation of Anabaena and Synechococcus, both in terms of fluctuations in the prey numbers and the development of the amoebal population, may partly be explained by differences in the size, growth form and reproductive rate of the two cyanobacterial species. Anabaena cells are relatively large (3x4um) and form long filaments, whereas those of Synechococcus are smaller (1x4um) and unicellular. These distinctions have a number of consequences. For example, the filamentous habit of Anabaena means that once a trichome is located a large number of cells can be ingested in a short space of time. Individual cells from fragmented trichomes are relatively easily located (by virtue of their size) and subsequently phagocytosed. These factors may explain both the rapid decrease and eventual elimination of the Anabaena population due to amoebal predation.

In contrast to Anabaena, the Synechococcus cells always occur individually. Although the initial chlorophyll a level was less for Synechococcus than for Anabaena, the number of cyanobacterial cells was many times greater. Acanthamoebae may be less efficient at locating and phagocytosing small prey, which would explain the slower rate of decline of the Synechococcus population.

Comparison of growth in the control flasks showed that the reproductive rate of Synechococcus was greater than that of the Anabaena. In the first 477h of incubation the population of

Synechococcus increased 4.9-fold, compared with 2.9-fold for Anabaena. The higher proliferation rate of Synechococcus, coupled with its small size, may explain the persistence of a residual population which avoided elimination by the amoebae.

Whilst the predation of Anabaena supported a small increase in amoebal population, no such increase occurred with Synechococcus. This may be because the initial population of Synechococcus cells was too low to support amoebal proliferation, or because predation only occurred at a rate which allowed maintenance of amoebae, but not reproduction. The persistence of relatively large numbers of trophozoites during the middle period of incubation of the Synechococcus cultures suggests that the residual Synechococcus population was capable of supporting a considerable amoebal biomass. Surprisingly, the marked increase in Synechococcus population during the latter stages of incubation was not accompanied by increases in the amoebal population.

In those flasks with Anabaena as the food source the elimination of the cyanobacterial population dictated that the amoebae had either to encyst or die through starvation. As an inevitable consequence of this, percentage encystment eventually reached 100%. Counts of the amoebal population showed that only a small proportion of the acanthamoebae underwent the transformation to cysts. Thus the increase in percentage encystment between 477h (27%) and 1049h (100%) was due largely to death of trophozoites rather than to recruitment to the cyst stage.

With Synechococcus as a food source the cyanobacterial population was never totally exhausted, with the result that

Fig.5.5. The effect of incubation temperature on the predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures.

Open symbols = experimental flasks (n=4) (initial amoebal population of $\triangleq 140 \times 10^3$ cells/ml)

Closed symbols = controls (n=2) (no amoebae added)

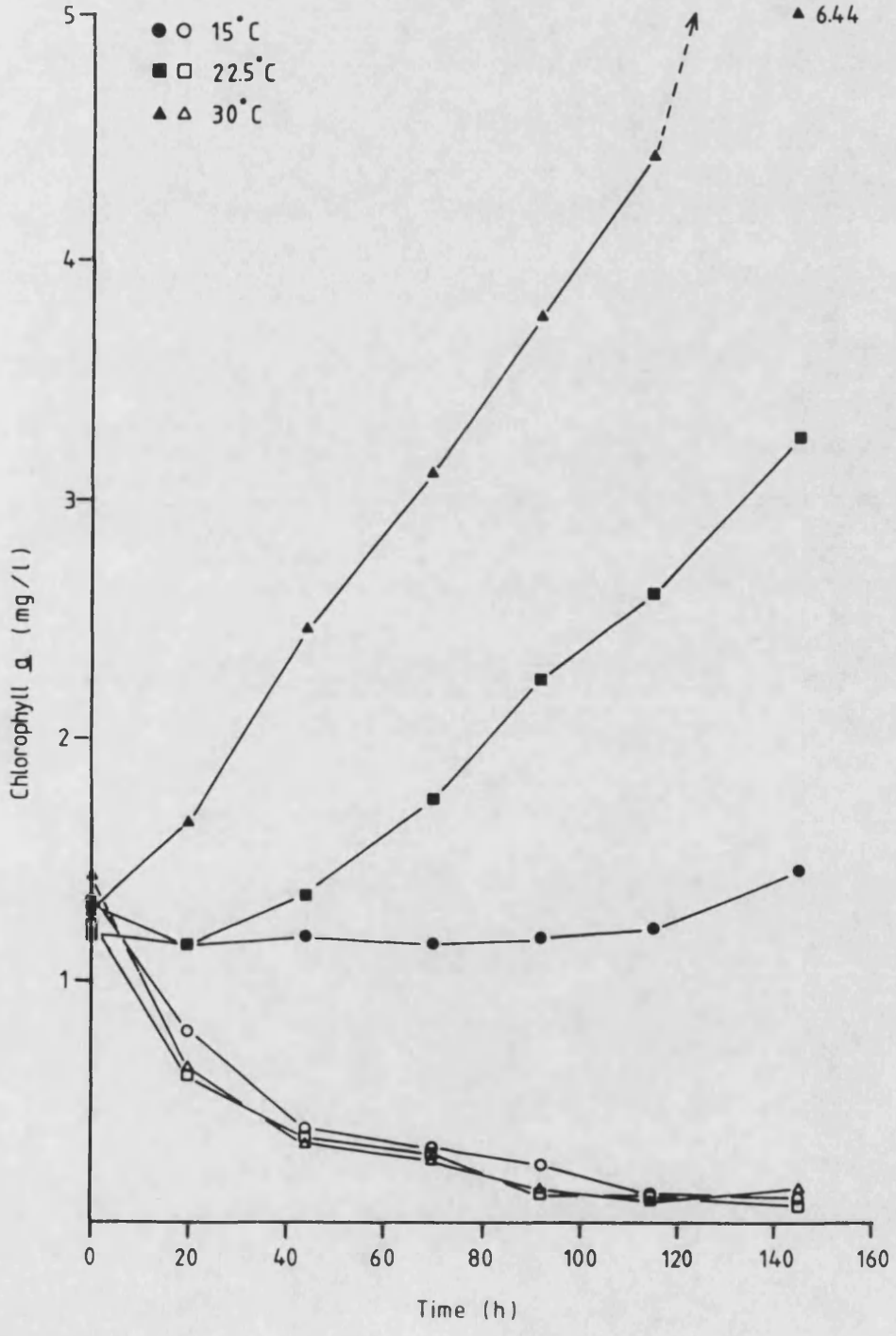


Fig.5.6. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures. Changes in trophozoite and cyst populations during incubation at three temperatures.
(All values are the mean of eight counts)

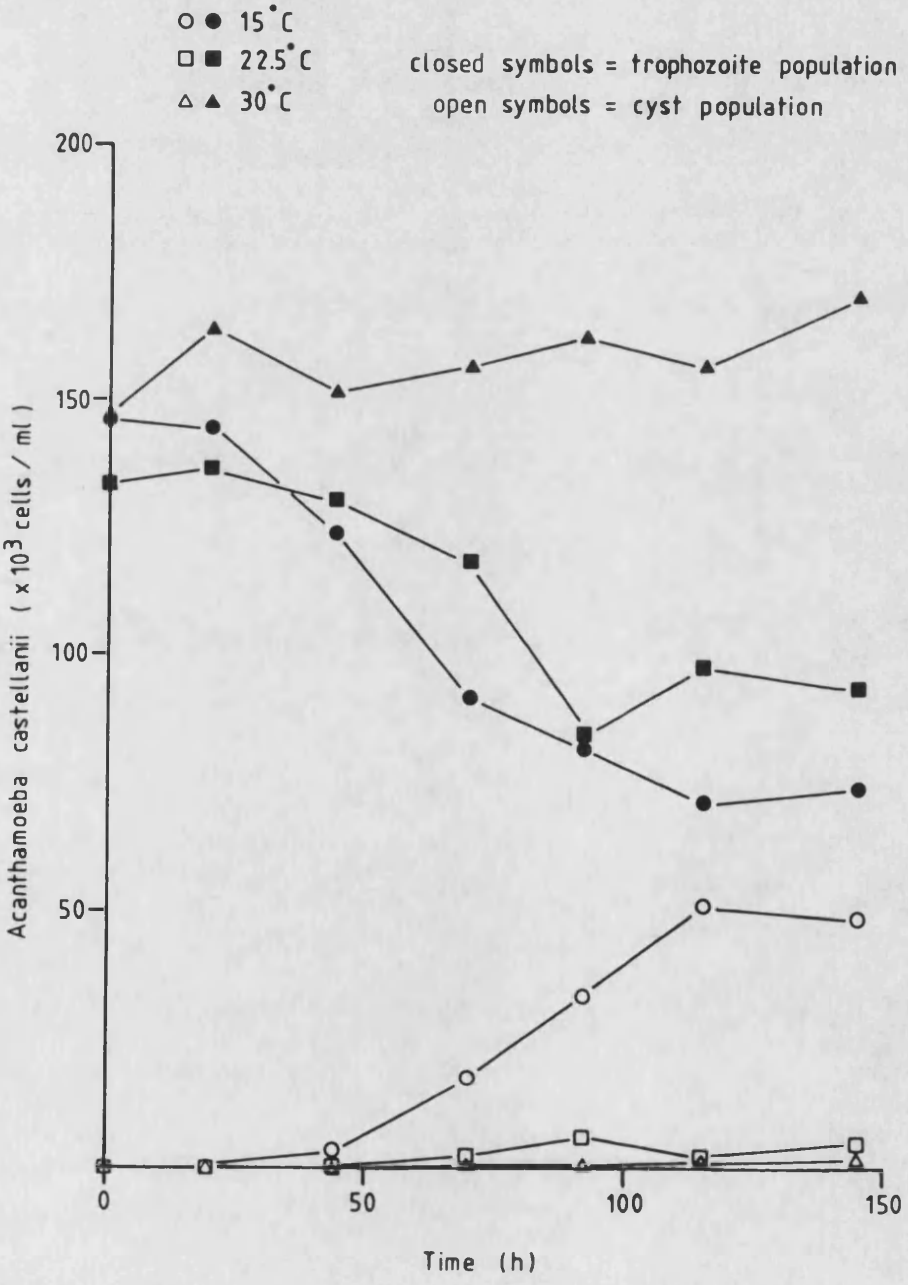


Table 5.2. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures. Changes in cyst (C) and trophozoite (T) numbers, total amoebal count (TAC), and the percentage of the Acanthamoeba population present as cysts (%E) for three incubation temperatures.

Time(h)	ACANTHAMOEBA CASTELLANII POPULATION ($x10^3$ cells/ml)*											
	15°C				22.5°C				30°C			
	T	C	TAC	%E	T	C	TAC	%E	T	C	TAC	%E
0	146.3 (11.0)	0 -	146.3 (11.0)	0	133.1 (4.4)	0 -	133.1 (4.4)	0	147.6 (6.0)	0 -	147.6 (6.0)	0
20	144.6 (5.7)	0.3 (0.2)	144.9 (5.6)	0.2	136.5 (5.9)	0.1 (0.1)	136.6 (5.9)	0.1	163.8 (4.5)	0.1 (0.1)	163.9 (4.5)	0.1
44	123.6 (9.7)	3.6 (1.8)	127.2 (10.9)	2.8	130.3 (5.7)	0.8 (0.8)	131.1 (5.2)	0.6	151.1 (5.6)	0.1 (0.1)	151.2 (5.5)	0.1
70	91.3 (6.5)	17.8 (7.7)	109.1 (13.0)	16.3	118.3 (7.7)	2.5 (0.9)	120.8 (7.5)	2.1	156.8 (7.3)	0.3 (0.2)	157.1 (7.4)	0.2
92	81.9 (11.9)	33.6 (7.2)	115.5 (15.2)	29.1	84.9 (5.9)	6.5 (2.7)	91.4 (8.0)	7.1	162.2 (7.6)	0.6 (0.4)	162.8 (7.6)	0.4
115	71.4 (7.2)	51.4 (14.2)	122.8 (18.1)	41.9	97.9 (2.9)	2.9 (0.8)	100.8 (2.9)	2.9	156.7 (3.5)	2.1 (1.3)	158.8 (4.5)	1.3
145	74.0 (7.2)	49.0 (8.4)	123.0 (12.9)	39.8	93.3 (4.5)	5.0 (1.5)	98.3 (5.7)	5.1	170.4 (6.5)	1.8 (0.8)	172.2 (6.7)	1.0

*Values are the mean of eight counts. Figures in parenthesis are +/- one standard error.

encystment was no longer the sole option ensuring amoebal survival. In consequence both the number of cysts and percentage encystment were lower than for Anabaena.

(B) The effect of temperature on predation

Results

The development of the Anabaena population at each of the incubation temperatures (15°C, 22.5°C and 30°C) is shown in Fig.5.5, and that of the trophozoite and cyst populations in Fig.5.6. Values for the total amoebal count and percentage encystment are given in Table 5.2.

Temperature had a strong effect on the growth of Anabaena in the control flasks, the extent of cyanobacterial proliferation increasing the higher the incubation temperature (Fig.5.5). The Anabaena population increased by 13%, 173% and 401% over the course of the experiment (145h) at 15°C, 22.5°C and 30°C respectively.

Amoebal predation resulted in the elimination of the Anabaena population at all three temperatures. The decline in cyanobacteria was similar in each case, but slightly more rapid at 22.5°C and 30°C than at 15°C. At each sampling the Anabaena population at 15°C was consistently greater than at the other temperatures. Thus after 20h incubation the Anabaena population had decreased by 40% at 15°C, 51% at 22.5°C and 55% at 30°C. Microscopic observation of samples after 70h showed that no Anabaena cells remained at 30°C and only a few at the two lower temperatures. After 115h the prey had been totally exhausted at all three temperatures (despite the small positive values obtained by chlorophyll extraction).

At 30°C the ingestion of Anabaena resulted in a modest increase

in the trophozoite population, numbers increasing from $148x10^3$ to $164x10^3$ cells/ml over the first 20h of incubation. Moreover, this population level was sustained for the duration of the experiment (Fig.5.6). This was in contrast to the two lower temperatures at which, apart from a small increase after 20h at 22.5°C, there was a progressive decline in trophozoite numbers. This decline was more marked at 15°C than at 22.5°C. After 145h trophozoite numbers were 115%, 70% and 51% of their initial level at 30°C, 22.5°C and 15°C respectively. Cyst numbers were negligible at the two higher temperatures, but rose sharply after 44h at 15°C, attaining a maximum population of $51x10^3$ cells/ml after 115h (percentage encystment of 42%) (see Table 5.2).

Discussion

Although the results indicate that predation of the Anabaena population by Acanthamoeba was more effective with increasing temperature, the observed differences between the three incubation temperatures were less marked than might have been expected, especially in view of the results obtained for predation in Repli dish cultures (Section two). A possible explanation for this may lie in the fact that the initial amoebal population in the flasks was very high in relation to the population of Anabaena. As a result of this excessive predator population, which led to a very rapid decrease in prey numbers, the full potential for differences in predation rate with incubation temperature may not have been realized.

Aside from the above comments, the amount of predation actually achieved at each temperature may be masked for entirely different

reasons. The Anabaena is controlled in each case despite the fact that it is increasing by division throughout the experiment, and that this proliferation is temperature-dependent (compare growth of controls, Fig.5.5). During the initial 44h of incubation there was a slight decrease in the prey population in the control flasks at 15°C, and a small increase at 22.5°C. Thus at these two temperatures there was no significant increase in the total number of Anabaena cells which would need to be ingested in order to control the cyanobacterial population. However, over the same time interval at 30°C the Anabaena population of the control flasks had almost doubled. If it is assumed that a similar rate of cyanobacterial proliferation took place in the experimental flasks, it is clear the amoebae are consuming many more cyanobacterial cells at 30°C than is at first apparent. Since the decrease in the level of the cyanobacterial population is similar to that at 15°C and 22.5°C, it follows that the rate of predation must be considerably higher at 30°C. Brabrand et al (1983) found that the predation rate of Nassula upon Oscillatoria was strongly influenced by temperature. Ingestion of Oscillatoria increased exponentially between 5 and 20°C. Oscillatoria populations were suppressed at all temperatures in this range.

The consumption of a greater number of Anabaena cells, in absolute terms, with increasing incubation temperature also provides an explanation for the observed differences in amoebal populations. Thus at 30°C, at which temperature the greatest number of Anabaena cells were consumed, predation resulted in an increase in the trophozoite population. By contrast, there was a steady decline in trophozoite numbers at 15°C, at which temperature the availability of

prey in absolute terms, and hence the extent of predation, was minimal. At 22.5°C the decrease in trophozoite population was less severe than at 15°C, reflecting a level of prey availability intermediate between that at 15°C and at 30°C.

The high values for percentage encystment at 15°C may be a consequence of this low availability of Anabaena cells in comparison to the other temperatures. Alternatively, the differences in percentage encystment may be a direct response by the amoebae to low temperature, rather than to the effects of temperature on prey availability.

(C) The effect of varying amoebal inoculum size

Results

Cell counts of the four Acanthamoeba inocula gave values of $8x10^3$, $24x10^3$, $52x10^3$ and $97x10^3$ cells/ml, hereafter referred to as inoculum levels 1, 2, 3 and 4 respectively. Development of the Acanthamoeba castellanii population from each inoculum level is shown in Fig.5.8. The Anabaena populations at each sampling are plotted in Fig.5.7, and values of trophozoite and cyst numbers, and percentage encystment, are listed in Table 5.3.

After an initial lag phase the control Anabaena population steadily increased, reaching a peak of 2.8mg/l chlorophyll a (an increase of 204% over the initial level) after 256h, before undergoing a gradual decline to 1.9mg/l chlorophyll a after 449h (Fig.5.7). In the experimental flasks the rate and extent of clearance of Anabaena A4 by Acanthamoeba increased the greater the amoebal inoculum. Thus clearance was first apparent with inoculum level 4, then with level 3

Fig.5.7. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures incubated at 15°C. The effect of amoebal inoculum size on the development of the cyanobacterial population. (Chlorophyll a levels are the mean from two extractions).

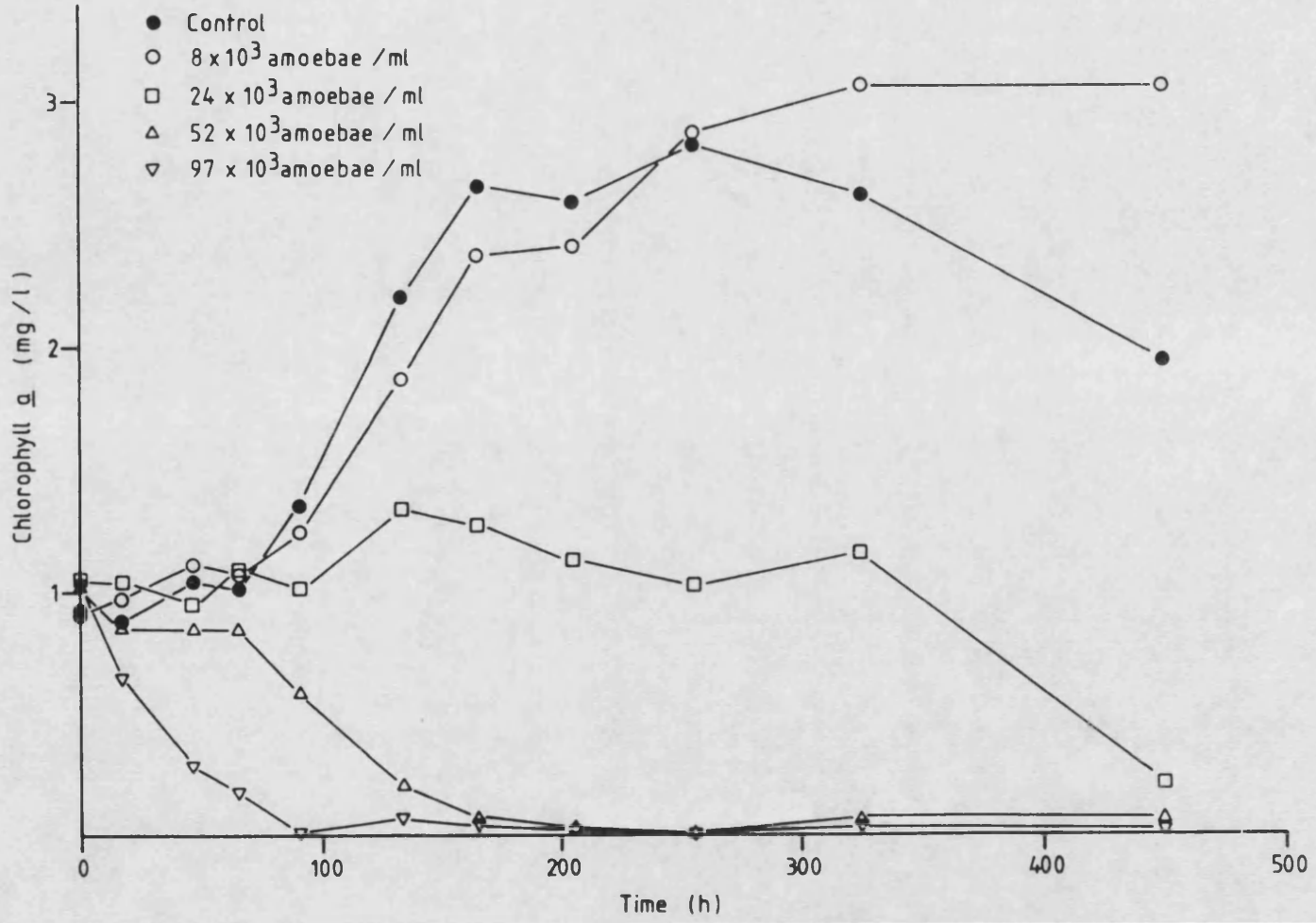


Fig.5.8. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures incubated at 15°C. The effect of Acanthamoeba inoculum density on the subsequent development of the amoebal population.

(All values are the mean of four counts).

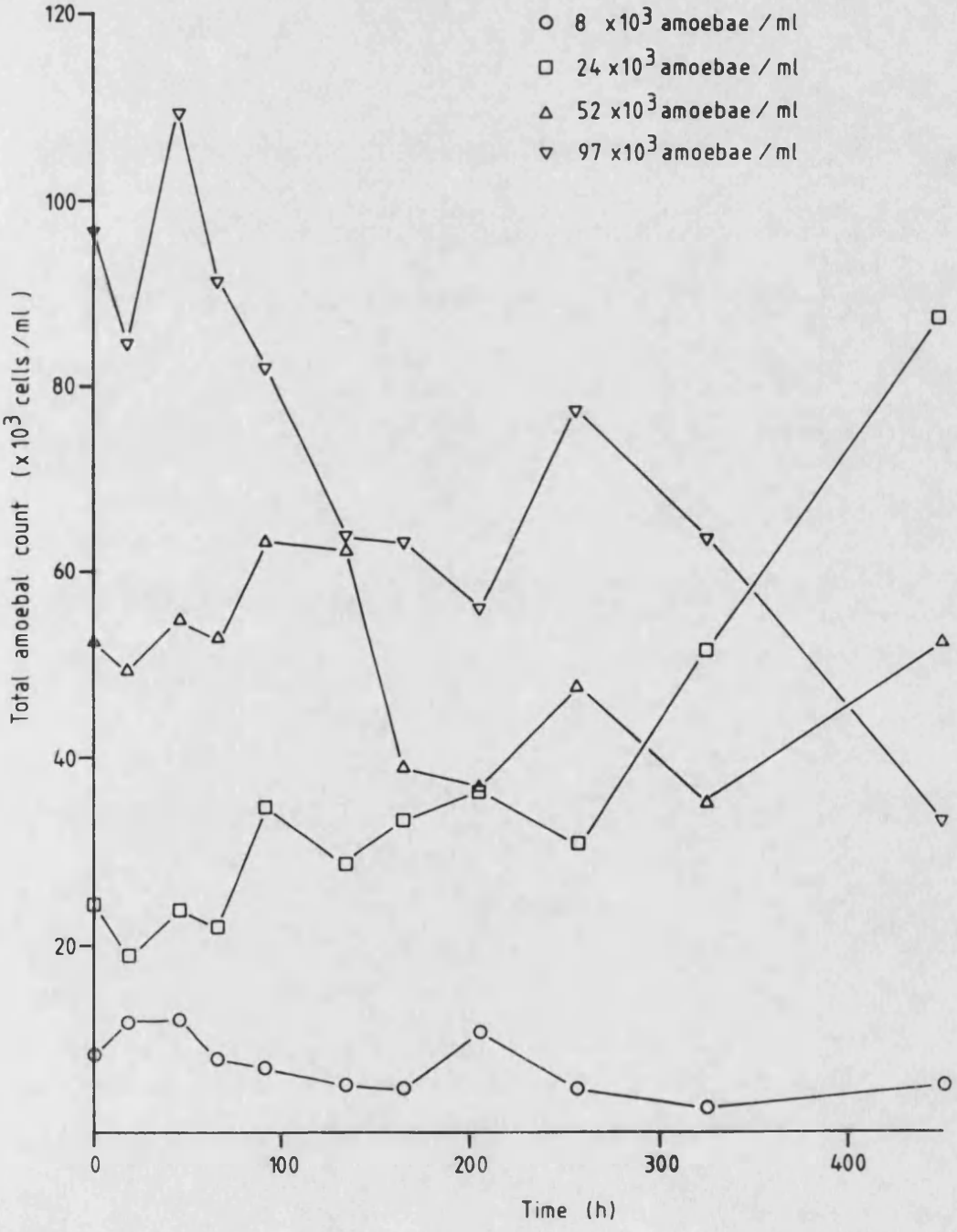


Table 5.3. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures (15°C). Changes in cyst* (C) and trophozoite* (T) numbers, total amoebal count* (TAC), and percentage encystment (%E) for four amoebal inoculum levels.

Time (h)	ACANTHAMOEBA INOCULUM DENSITY													
	$8x10^3$ cells/ml							$24x10^3$ cells/ml						
	T		C		TAC		%E	T		C		TAC		%E
0	8.3	(0.7)	0	-	8.3	(0.7)	0	24.4	(0.5)	0	-	24.4	(0.5)	0
18	11.7	(1.0)	0	-	11.7	(1.0)	0	18.9	(2.1)	0.3	(0.3)	19.2	(2.1)	1.6
47	11.4	(1.9)	0.6	(0.6)	12.0	(2.4)	5.0	18.1	(1.7)	5.6	(2.1)	23.7	(3.7)	23.6
66	5.0	(1.2)	2.8	(1.0)	7.8	(1.5)	35.9	10.8	(2.2)	10.8	(3.7)	21.6	(4.8)	50.0
91	3.6	(0.8)	3.3	(0.9)	6.9	(1.1)	47.8	16.1	(2.5)	18.6	(2.5)	34.7	(3.8)	53.6
134	4.2	(0.5)	0.8	(0.3)	5.0	(0.6)	16.0	22.8	(1.7)	5.8	(1.8)	28.6	(1.5)	20.3
165	1.9	(0.8)	2.8	(1.2)	4.7	(1.2)	59.6	24.2	(3.4)	8.9	(2.9)	33.1	(4.2)	26.9
205	3.1	(0.5)	7.5	(0.5)	10.6	(1.0)	70.8	26.1	(8.0)	10.3	(2.3)	36.4	(7.7)	28.3
256	0.3	(0.3)	4.4	(1.6)	4.7	(1.7)	93.6	18.6	(9.6)	12.2	(4.0)	30.8	(12.7)	39.6
325	0.3	(0.3)	2.5	(0.5)	2.8	(0.6)	89.3	15.6	(5.3)	35.8	(5.8)	51.4	(10.9)	69.6
449	0.3	(0.3)	4.7	(1.8)	5.0	(1.7)	94.0	42.5	(10.2)	45.0	(3.1)	87.5	(7.6)	51.4

* Values are the mean of four counts. All values for amoebal populations are $x10^3$ cells/ml. Figures in parenthesis are

Table 5.3 (continued)

Time (h)	ACANTHAMOEBA INOCULUM DENSITY													
	$52x10^3$ cells/ml						$97x10^3$ cells/ml							
	T		C		TAC		%E	T		C		TAC		%E
0	52.2	(2.8)	0	-	52.2	(2.8)	0	96.7	(4.7)	0	-	96.7	(4.7)	0
18	49.2	(3.1)	0	-	49.2	(3.1)	0	84.2	(3.0)	0.3	(0.3)	84.5	(3.1)	0.4
47	39.4	(5.8)	15.3	(4.5)	54.7	(5.7)	28.0	99.7	(5.4)	9.7	(4.5)	109.4	(9.5)	8.9
66	32.8	(7.3)	20.0	(5.7)	52.8	(8.8)	37.9	73.6	(7.0)	17.8	(6.7)	91.4	(11.8)	19.6
91	42.5	(1.8)	20.8	(2.2)	63.3	(3.4)	32.9	46.7	(4.2)	35.3	(6.5)	82.0	(6.3)	43.0
134	44.7	(2.6)	17.5	(4.1)	62.2	(5.1)	28.1	34.4	(1.5)	29.4	(10.9)	63.8	(10.3)	46.1
165	28.3	(4.6)	10.6	(2.1)	38.9	(4.0)	27.2	32.5	(4.0)	30.6	(8.6)	63.1	(5.8)	48.5
205	19.7	(3.1)	16.9	(2.5)	36.6	(2.1)	46.2	21.4	(2.8)	34.4	(10.6)	55.8	(13.3)	61.6
256	20.3	(4.3)	27.2	(2.9)	47.5	(4.5)	57.3	33.6	(4.5)	43.9	(12.5)	77.5	(12.3)	56.6
325	15.8	(2.3)	19.2	(3.0)	35.0	(5.0)	54.9	17.5	(3.6)	45.6	(7.3)	63.1	(8.9)	72.3
449	11.7	(1.3)	40.6	(2.8)	52.3	(1.6)	77.6	3.6	(1.1)	29.7	(4.0)	33.3	(3.5)	89.2

* Values are the mean of four counts. All values for amoebal populations are $x10^3$ cells/ml. Figures in parenthesis are +/- one standard error.

and finally with level 2. With inoculum level 1 amoebal predation was unable to control the Anabaena population, which increased throughout the duration of the experiment, eventually attaining a level three times greater than the initial population and greater than that of the control (Fig.5.7). After 91h incubation the Anabaena population had increased by 38% with amoebal inoculum 1, and had decreased by 5%, 41%, and 99% at levels 2, 3 and 4, respectively (Fig.5.7). These values were equivalent to 92%, 75%, 44% and 1%, respectively, of the control Anabaena population.

Despite considerable variability in the data for the plots of total amoebal count (Fig.5.8), certain general trends were discernable. With inoculum level 1 there was a gradual decline in the Acanthamoeba population. With level 2, by contrast, there was a small increase in amoebae over the first 250h of incubation, followed by large increases at the last two samplings. Between 256h and 449h the Acanthamoeba population increased from 30.8×10^3 to 87.5×10^3 cells/ml (Table 5.3). In those flasks with inoculum level 3 the number of amoebae rose by approximately 10×10^3 cells/ml to 63.3×10^3 cells/ml after 91h, before falling and levelling off at between 40×10^3 and 50×10^3 cells/ml for the remainder of the experiment. With level 4 there was also a small initial rise in the amoebal population, after which numbers steadily declined. By 449h numbers were reduced to a third of their initial level (Fig.5.8).

Encystment was first recorded after 18h for inoculum levels 2 and 4, and after 47h for levels 1 and 3. Values for percentage encystment (%E) showed an upwards trend with successive samplings (best seen for inoculum level 4 - Table 5.3). After 449h %E was 94%,

78% and 89% for levels 1, 3 and 4, respectively, and considerably less (51%) for level 2.

Discussion

These results demonstrate that the ability of Acanthamoeba castellanii to eliminate Anabaena A4 from batch cultures, and the rate at which such clearance is achieved, is to a major extent a function of the size of the amoebal inoculum. Thus the lowest amoebal inoculum ($8x10^3$ cells/ml - level 1) was unable to control the cyanobacterial growth, which increased steadily throughout the duration of the experiment (449h). In contrast, the Anabaena populations were virtually eliminated by the two highest levels of amoebal inoculum ($52x10^3$ cells/ml (level 3) and $97x10^3$ cells/ml (level 4)) after 200h. For inoculum level 2 ($24x10^3$ cells/ml) the values plotted in Fig.5.7 indicate an intermediate position in which the Anabaena population is effectively reduced, but only after an extended period of incubation. However, this conclusion is partially misleading in that, unlike the three other amoebal inoculum levels, the development of predator and prey populations in the two flasks with inoculum level B were markedly different (each point in Fig.5.7 is the mean of two values, one from each flask). In one flask the Anabaena population remained stable for the first 134h of incubation, and then decreased rapidly to 12% of its original level by 256h. By 449h Anabaena numbers were only 1% of their initial level. In the second flask the Anabaena population increased by 104% during the first 325h, followed by a rapid decline (38% of the initial level after 449h). The Acanthamoeba population in this flask increased from $36x10^3$ to $101x10^3$ cells/ml between 325 and 449h

incubation.

Therefore, whilst the end result (reduction in Anabaena population) was the same for both flasks, the way in which it was achieved was different in each case. On the one hand amoebal predation checked the growth in prey population from the outset, and then reduced it, whilst on the other the Anabaena increased substantially before predation by Acanthamoeba could exert effective control.

These differing responses in the two flasks may indicate that the balance of predator and prey at this amoebal inoculum level is such that there is equal liklihood of two alternative outcomes. Either proliferation of Anabaena outstrips the rate at which it is predated by amoebae (leading to an increase in prey population), or the reverse situation prevails in which predation by amoebae exceeds the rate of cyanobacterial proliferation (leading to the reduction, and eventual elimination, of Anabaena). The existence of such a "threshold" is supported by the experimental evidence in that complete clearing of Anabaena occurred with those inoculum levels where the ratio of predator to prey was shifted in favour of the amoebae (levels 3 and 4), whereas with inoculum level 1, in which the ratio was shifted in favour of the cyanobacterium, there was an increase in the Anabaena population. It would be desirable to repeat these experiments with greater replication and with amoebal inoculum densities nearer (both above and below) that of level 2, in order to more accurtely determine the position of this threshold.

The size of the initial Anabaena population only permitted modest rises in the total amoebal count with the two highest amoebal inocula. As expected, exhaustion of the prey led to a reduction in

total amoebal count (TAC) and an increase in percentage encystment (%E). With inoculum level 2, the sharp increase in TAC towards the end of the experiment resulted from the predation of the Anabaena population which had developed in one of the flasks (see above). The occurrence of actively predating trophozoites at this inoculum level during the latter part of the experiment explains the relatively low value (51%) for %E after 449h. The absence of any appreciable increase in predator population with inoculum level 1 was surprising, especially in view of the fact that the availability of food, in relation to the number of amoebae present, was greatest at this level.

(D) The effect of varying cyanobacterial inoculum size

Results

Development of the Anabaena population from each of the four inoculum levels is shown in Fig.5.9. The total amoebal counts at each sampling are plotted in Fig.5.10, and values of trophozoite and cyst numbers, and percentage encystment, are listed in Table 5.4.

Chlorophyll a levels for the initial samples (0h) gave values for the four Anabaena inocula of 0.87, 1.85, 2.48 and 3.29 mg/l, hereafter referred to as inoculum levels A, B, C and D respectively. After an initial lag phase there was a steady increase in control Anabaena populations for each inoculum level (Fig.5.9). By contrast, predation by Acanthamoeba in the experimental flasks had, after 169h incubation, virtually eliminated the cyanobacteria in all cases. During the first 44h of incubation the Anabaena population declined by 74.9%, 71.2%, 57.4% and 57.1% (decreases in chlorophyll a concentration of 0.68, 1.38, 1.41 and 1.88 mg/l) for inoculum levels

Fig.5.9. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures (20°C). The effect of the initial Anabaena population density on the ability of amoebae to control subsequent cyanobacterial proliferation.

Open symbols = controls (minus amoebae) (n=2)

Closed symbols = experimentals (plus amoebae) (n=3)

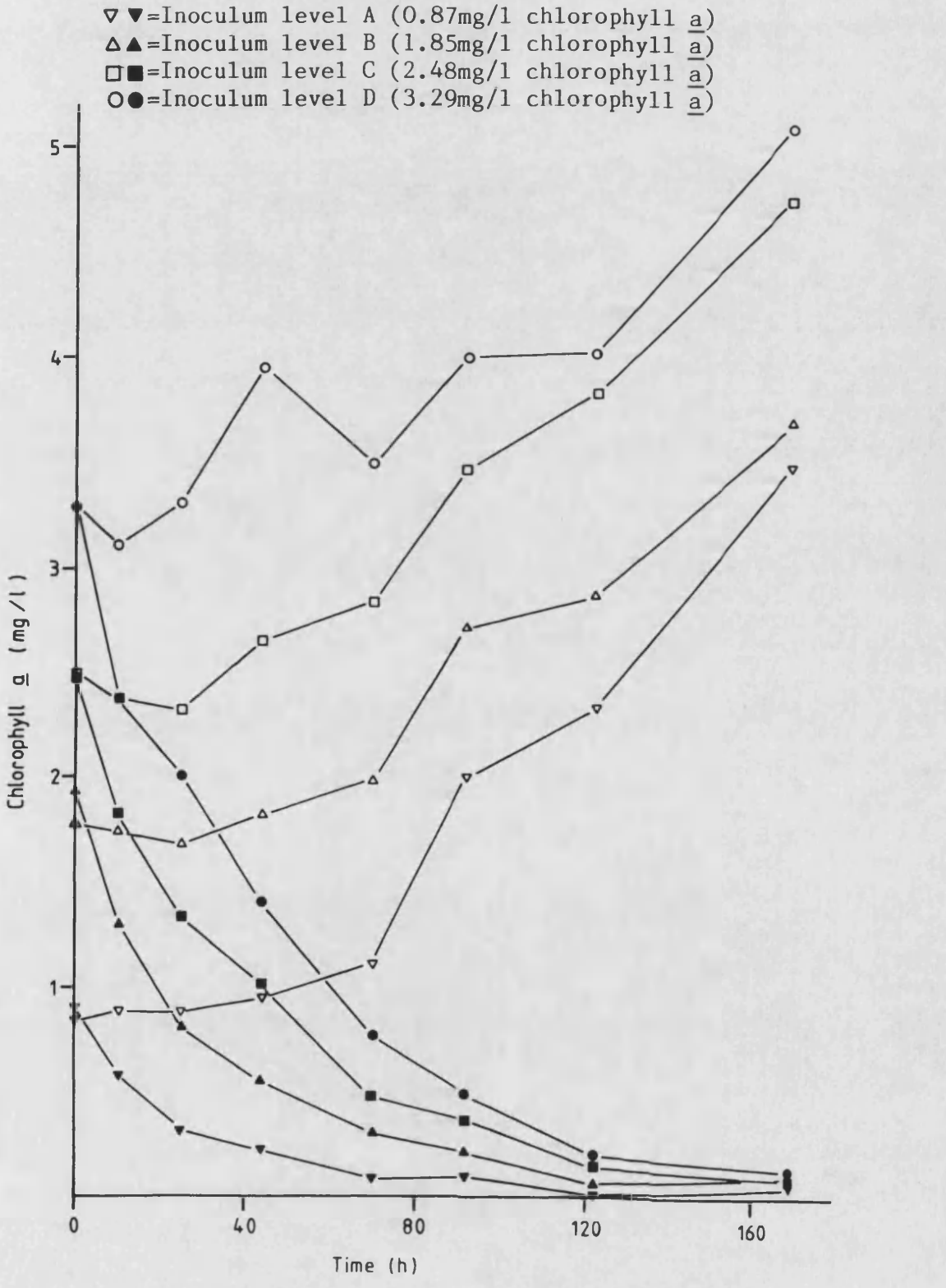


Fig.5.10. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures (20°C). The effect of cyanobacterial inoculum density on the development of the amoebal population.

▽ = Inoculum level A (0.87mg/l chlorophyll a)

△ = Inoculum level B (1.85mg/l chlorophyll a)

□ = Inoculum level C (2.48mg/l chlorophyll a)

○ = Inoculum level D (3.29mg/l chlorophyll a)

(All values for the Acanthamoeba population are the mean of six counts)

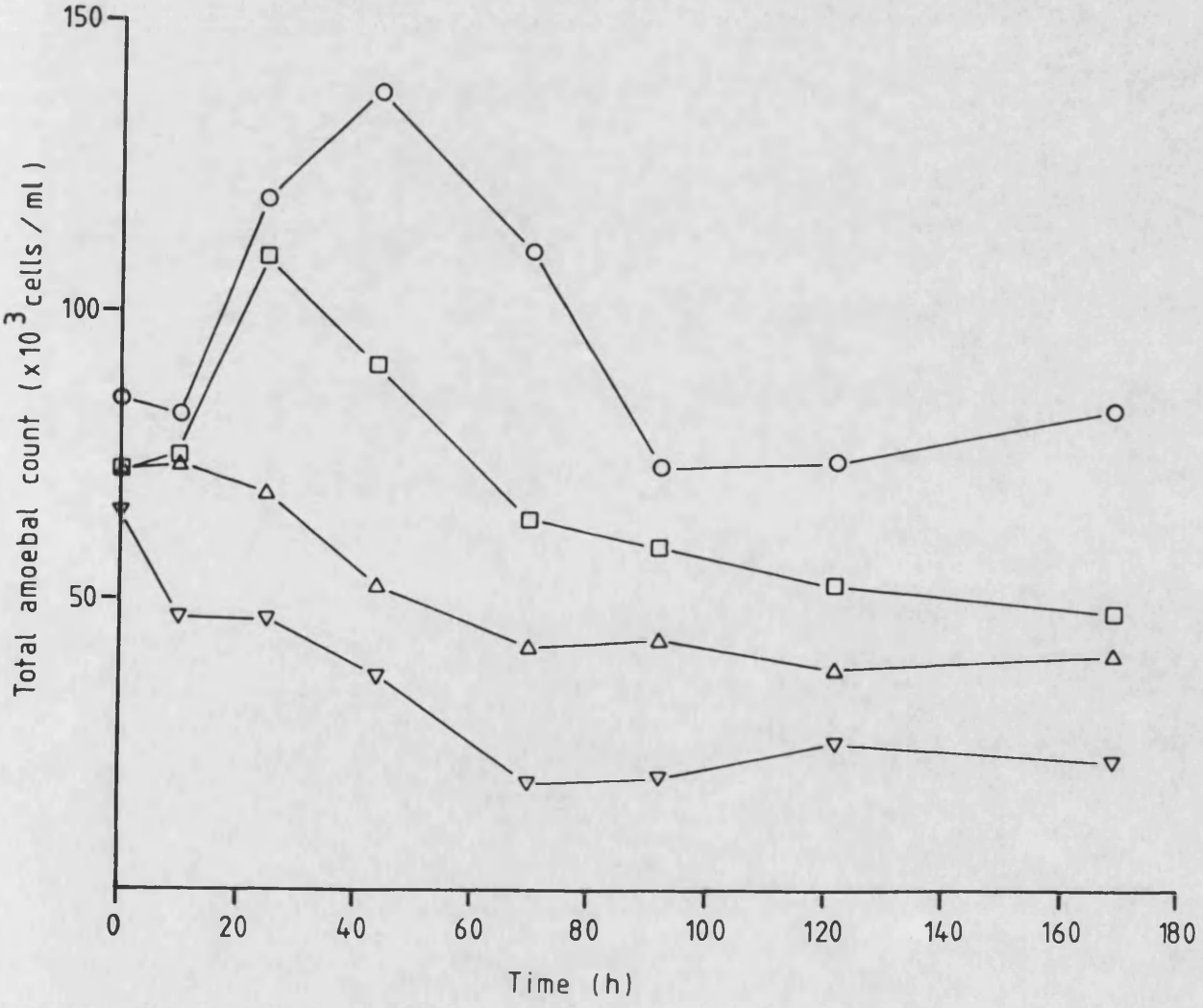


Table 5.4. Predation of Anabaena A4 by Acanthamoeba castellanii PB in illuminated batch cultures (20°C). Variation in cyst* (C) and trophozoite* (T) numbers, total amoebal count* (TAC), and percentage encystment (%E) with incubation time for four cyanobacterial inoculum levels.

Time(h)	CYANOBACTERIAL INOCULUM LEVEL													
	Level A							Level B						
	T		C		TAC		%E	T		C		TAC		%E
0	65.0	(4.6)	0	-	65.0	(4.6)	0	71.9	(2.5)	0	-	71.9	(2.5)	0
10	47.4	(3.0)	0	-	47.4	(3.0)	0	72.4	(6.0)	0	-	72.4	(6.0)	0
25	45.9	(4.2)	0.9	(0.9)	46.8	(4.9)	1.9	67.4	(3.8)	0.4	(0.2)	67.8	(3.9)	0.6
44	35.6	(3.1)	1.1	(1.1)	36.7	(2.9)	3.0	51.7	(4.8)	0	-	51.7	(4.8)	0
70	17.0	(2.2)	1.9	(1.9)	18.9	(3.6)	10.1	39.1	(3.3)	2.4	(1.2)	41.5	(4.4)	5.8
92	19.1	(2.9)	0.6	(0.4)	19.7	(3.0)	3.0	33.7	(2.8)	9.1	(5.4)	42.8	(6.9)	21.3
122	22.4	(2.1)	3.0	(2.5)	25.4	(2.5)	11.8	32.0	(3.7)	5.2	(4.5)	37.2	(4.2)	14.0
169	20.4	(2.6)	1.7	(1.1)	22.1	(3.3)	7.7	32.8	(3.4)	7.2	(1.7)	40.0	(4.4)	18.0
504	0.8	(0.4)	2.8	(0.8)	3.6	(0.8)	77.8	0	-	14.4	(3.5)	14.4	(3.5)	100.0

* All values for amoebal numbers are $x10^3$ cells/ml. Each value is the mean of six counts. Figures in parenthesis are +/- one standard error.

(Continued)

Table 5.4 (continued)

Time(h)	CYANOBACTERIAL INOCULUM LEVEL													
	Level C							Level D						
	T		C		TAC		%E	T		C		TAC		%E
0	71.9	(2.7)	0	-	71.9	(2.7)	0	84.7	(4.8)	0	-	84.7	(4.8)	0
10	74.2	(6.0)	0	-	74.2	(6.0)	0	81.7	(6.2)	0	-	81.7	(6.2)	0
25	108.0	(5.8)	1.1	(0.3)	109.1	(5.9)	1.0	118.7	(5.4)	0.4	(0.4)	119.1	(5.4)	0.3
44	87.0	(4.6)	3.3	(2.1)	90.3	(6.4)	3.7	135.8	(3.1)	2.0	(1.8)	137.8	(3.9)	1.5
70	59.1	(3.6)	4.3	(2.3)	63.4	(4.7)	6.8	103.7	(8.3)	6.5	(3.2)	110.2	(10.0)	5.9
92	52.0	(7.2)	6.7	(3.0)	58.7	(7.0)	11.4	68.9	(4.2)	3.5	(1.1)	72.4	(4.3)	4.8
122	46.4	(3.9)	5.9	(1.8)	52.3	(5.2)	11.3	57.8	(4.7)	15.9	(3.3)	73.7	(5.3)	21.6
169	40.2	(2.9)	7.0	(1.6)	47.2	(2.9)	14.8	55.6	(4.5)	26.5	(7.5)	82.1	(9.6)	32.3
504	0.2	(0.2)	15.2	(2.5)	15.4	(2.6)	98.7	0	-	40.8	(4.9)	40.8	(4.9)	100.0

All values for amoebal numbers are $x10^3$ cells/ml. Each value is the mean of six counts. Figures in parenthesis are +/- one standard error. (See text for explanation of cyanobacterial inoculum levels)

A, B, C and D respectively. The rate of decline of the Anabaena populations at all four inoculum levels decreased during the course of the experiment (Fig.5.9).

Marked differences occurred in the development of the Acanthamoeba population at each inoculum level (Fig.5.10). For inoculum levels A and B there was a steady decline in total amoebal count (TAC) with incubation time, whilst with levels C and D there was an initial increase in amoebal numbers, followed by a decline. Thus after 44h incubation the Acanthamoeba population had decreased by 44% at level A and by 28% at level B, but had increased by 26% at level C and by 63% at level D. Maximal amoebal numbers for the latter two inoculum levels were 109×10^3 cells/ml for level C (25h) and 137.8×10^3 cells/ml for level D (44h).

Both the cyst population in absolute terms and percentage encystment increased with incubation for all four inoculum levels (Table 5.4). After extended incubation (504h) the cyst populations were 2.8, 14.4, 15.2 and 40.8×10^3 cells/ml at inoculum levels A, B, C and D respectively, with values for percentage encystment high in each instance (100% for levels B and D).

Discussion

As in the previous experiment, predation by amoebae led to the effective control of the Anabaena population. With the highest cyanobacterial inoculum level (level D) the initial Anabaena population was six times more abundant, in relation to the numbers of Acanthamoeba present, than was the case for the previous investigations on the effect of temperature on predation. For inoculum

levels C and D the period of incubation during which amoebal proliferation took place was concomitant with a sharp decline in the cyanobacterial population - the energy obtained by trophozoites feeding on the Anabaena cells was rapidly expressed in the production of new amoebal biomass. As expected, exhaustion of the food source (at all four inoculum levels) was followed by a slow decline in trophozoite numbers and an associated increase in the cyst population.

A possible explanation for the decline in the rate of predation of the Anabaena populations during the course of incubation is that the cyanobacterial food pool becomes limiting as it diminishes in size. Amoebal trophozoites encounter the remaining Anabaena cells at a frequency lower than their capacity to endocytose new cells, and in consequence the predation rate declines.

The differences in development of the Acanthamoeba populations at the four inoculum levels were directly attributable to the differences in the availability of cyanobacterial prey. At inoculum levels A and B there were too few Anabaena cells to support proliferation of trophozoites, resulting in the observed decrease in total amoebal count (Fig.5.10). This decline was, as expected, more pronounced for inoculum level A since cyanobacterial numbers were minimal at this level. The more abundant food supply at levels C and D was able to support amoebal reproduction, the highest Acanthamoeba population developing at level D, which had the greatest prey numbers.

SECTION FOUR

TRANSMISSION ELECTRON MICROSCOPY

Introduction

The internalization of cyanobacterial food particles through phagocytosis is only one stage in the feeding process of Acanthamoeba. Once prey has been ingested it must be digested to enable release of nutrients and energy for amoebal growth and reproduction. This section presents some ultrastructural observations on the digestion of cyanobacterial cells by Acanthamoeba castellanii.

Materials and Methods

Treatment of specimens prior to fixing

(a) Non-feeding acanthamoebae

Samples (4ml) from a 7d statically grown culture (A. castellanii NEFF G) were transferred to test tubes and the cells sedimented by centrifugation (5min at 500g). Most of the supernatant was removed, and the amoebae left for 20min to regain their normal cell shape prior to the addition of fixative.

(b) Mixed populations of predator and prey

With Synechococcus leopoliensis 1405/1 and Plectonema boryanum DAFT 594 as prey, 2ml of an established batch culture of the cyanobacterium was mixed in a test tube with an equal volume of amoebal culture (A. castellanii PB, growth conditions as above). Tubes

were incubated overnight in the light (Photosynthetically active radiation of 45 microeinsteins $m^{-2}s^{-1}$) at 30°C. In the case of Anabaena A4, samples (1ml) of cyanobacterial culture were dispensed into Repli dish wells together with 1ml of amoebal suspension. The amoebal suspension was prepared by centrifugation of a 5ml sample of A. castellanii PB from a 2d batch culture, removal of the supernatant, and resuspension of cells in 100ml sterile Allen's medium. Mixed cyanobacterial/amoebal cultures were incubated statically in the dark for 4h at 20°C prior to fixation. The cells in all amoebal/cyanobacterial mixed cultures settled out during incubation. Most of the supernatant in the tubes/wells was removed by Pasteur pipette before adding fixative.

Fixation and subsequent preparation

Cells were fixed in 3% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH 6.8. 1mM calcium chloride was incorporated into the buffer to enhance the preservation of membranes. Cells were fixed for 2.5h at 4°C, all chemicals having been cooled prior to usage. Fixed cells were transferred to centrifuge tubes (if not already in tubes) and washed twice in the cacodylate buffer containing 1mM calcium chloride (first wash 5min, second 10min), the cells being pelleted by centrifugation (5min at 500g) prior to each change of solution. Cells were postfixed for 1h in 1% (v/v) osmium tetroxide in the same buffer without calcium chloride, followed by two washes in distilled water. Washed, pelleted cells were mixed in a Pasteur pipette with molten 2% agarose agar at just above setting temperature. Small drops of agar containing suspended cells of amoebae and

cyanobacteria were extruded onto the plastic base of a Petri dish and left to solidify. Agar blocks with embedded material were transferred to glass vials and dehydrated by passage through an ethanol series (50%, 70%, 75%, 90%, 95%, 100%, 100%, 100%) followed by three changes in propylene oxide, with 15 min in each solution. Specimens were left overnight in a mixture of 50% propylene oxide and 50% Taab E.M. embedding resin (Taab Laboratries, Aldermaston, Berks.), and then transferred to 100% resin with three changes over two days. The vials were placed on a mechanical rotator to increase the rate of impregnation of the specimen with resin. Single agar blocks were placed in the compartments of a mould containing fresh resin, and left to polymerize in an oven at 60°C for 48h.

Silver-grey sections were cut from trimmed blocks using an OM3 ultramicrotome (Reichert-Jung, Cambridge) with glass knives, and collected from water on uncoated 200 mesh copper grids. Sections were stained first in uranyl acetate (saturated solution in 70% ethanol) for 7min, followed by Reynolds lead citrate for 10min. Specimens were viewed with a Jeol 100CX transmission electron microscope (Jeol U.K. Ltd., Colindale, London) operated at 80KV.

Results

(1) General morphology

The general morphology of trophozoites was the same for both the *A. castellanii* strains investigated (NEFF G and PB) (see Fig.6.1 A). Cells were bounded by a plasma membrane and were more or less rounded in outline. Most sections contained a few fine pseudopodial projections (acanthopodia), the outer membrane of which was continuous

Transmission electron microscopy figures

(All micrographs, with the exception of Fig.6.1 A, are of A. castellanii PB)

Fig.6.1

(A) General organization of a non-feeding trophozoite of A. castellanii G. (N = nucleus, Nu = nucleolus, V = vacuole, M = mitochondrion, Ac = acanthopodium). (Bar marker = 4μm)

(B) Trophozoite containing Anabaena A4 cells in food vacuoles at various stages of digestion (amoebae were fixed 4h after introduction of the cyanobacterial food source). (V1 = food vacuole with newly ingested Anabaena cells. V2 = food vacuole with Anabaena cells at early stages of digestion. V3 = old food vacuole in which the cellular integrity of Anabaena has been totally lost. (Bar marker = 4μm)

Fig.6.2

(A) Higher magnification view of part of Fig.6.1 B. (An = Anabaena A4 cell, FVM = food vacuole membrane). The plane of section has passed through the cytoplasmic connection (arrowed) between two cells of the Anabaena filament. (Bar marker = 2μm)

(B) Section of an Anabaena A4 cell which has not been phagocytosed (i.e. one which is free in the surrounding medium). (W = cell wall, Th = thylakoids, G = dense granule). (Bar marker = 0.5μm).

Fig.6.3

(A) Section through an Anabaena A4 cell within an amoebal food vacuole. Part of the cyanobacterial cell wall is showing evidence of disruption (arrowed). (An = Anabaena, L = lipid droplet, M = mitochondrion, C = cristae, FV = food vacuole). (Bar marker = 1μm)

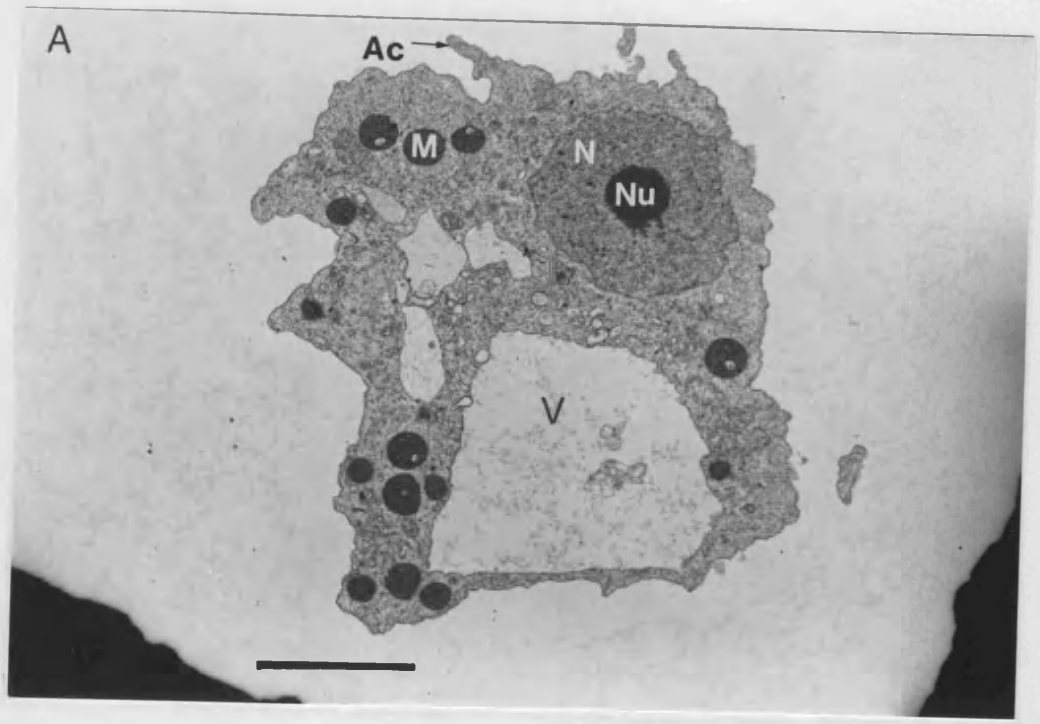
(B) Trophozoite with three food vacuoles (V) containing Anabaena cells at an advanced stage of digestion. (Bar marker = 2μm).

Fig.6.4

(A) Section showing an old food vacuole in a trophozoite fed Plectonema boryanum. The only visible remains of the cyanobacteria are dispersed thylakoid membranes (TH). (CV = cntractile vacuole). (Bar marker =3μm)

(B) Trophozoite containing newly ingested cells of Synechococcus leopoliensis (S). Each cell is individually enclosed in a food vacuole. (Bar marker = 2μm).

Fig. 6.1



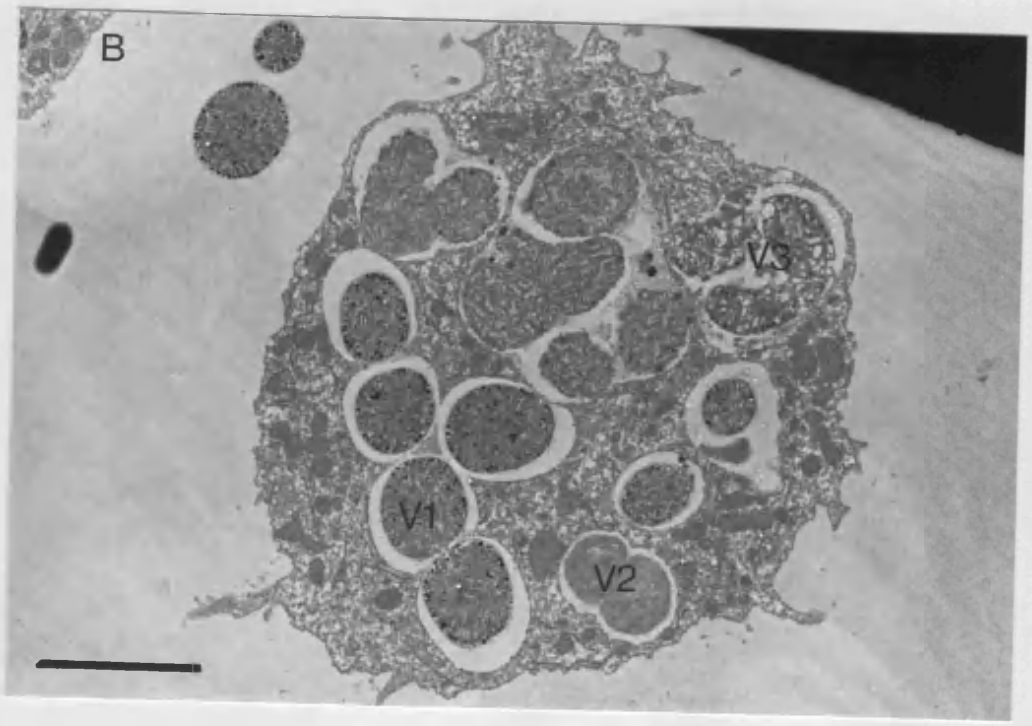
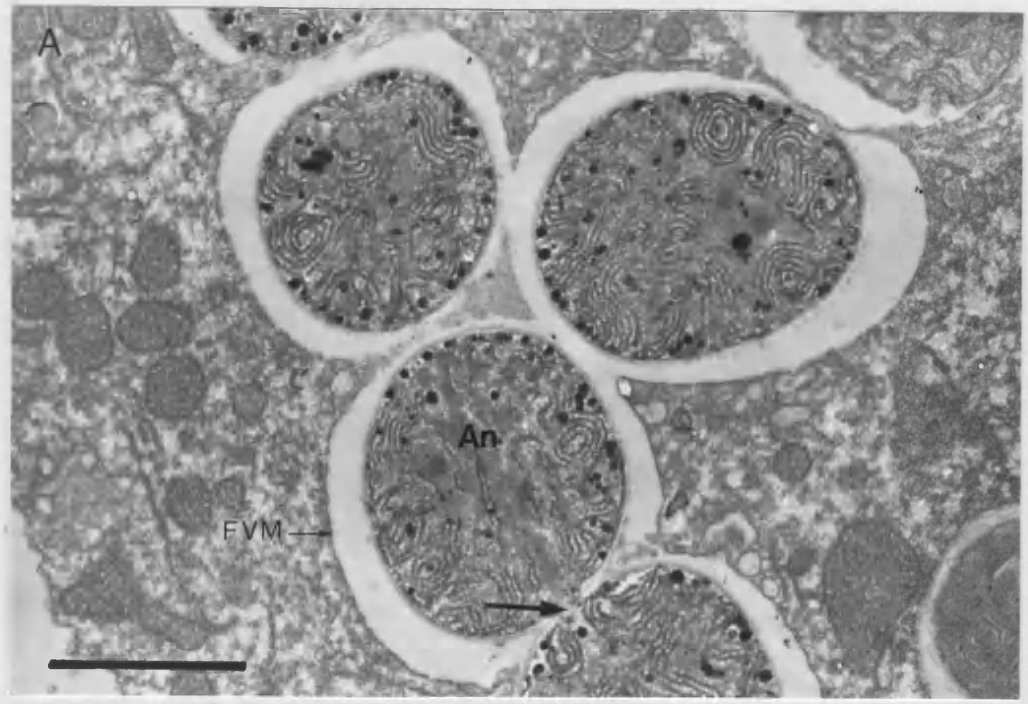


Fig. 6.2



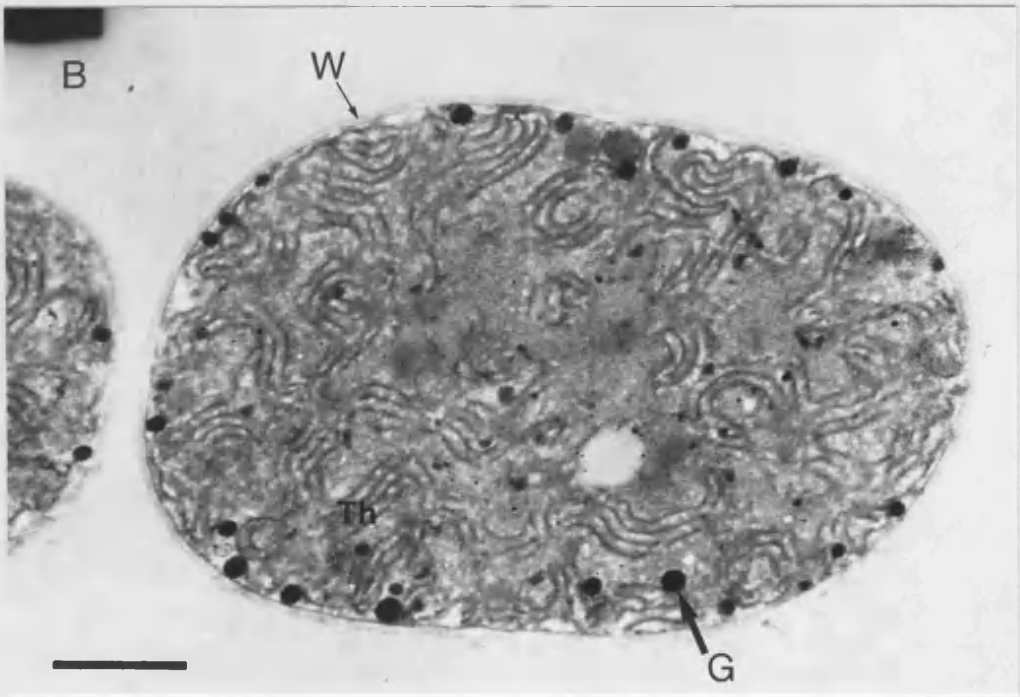
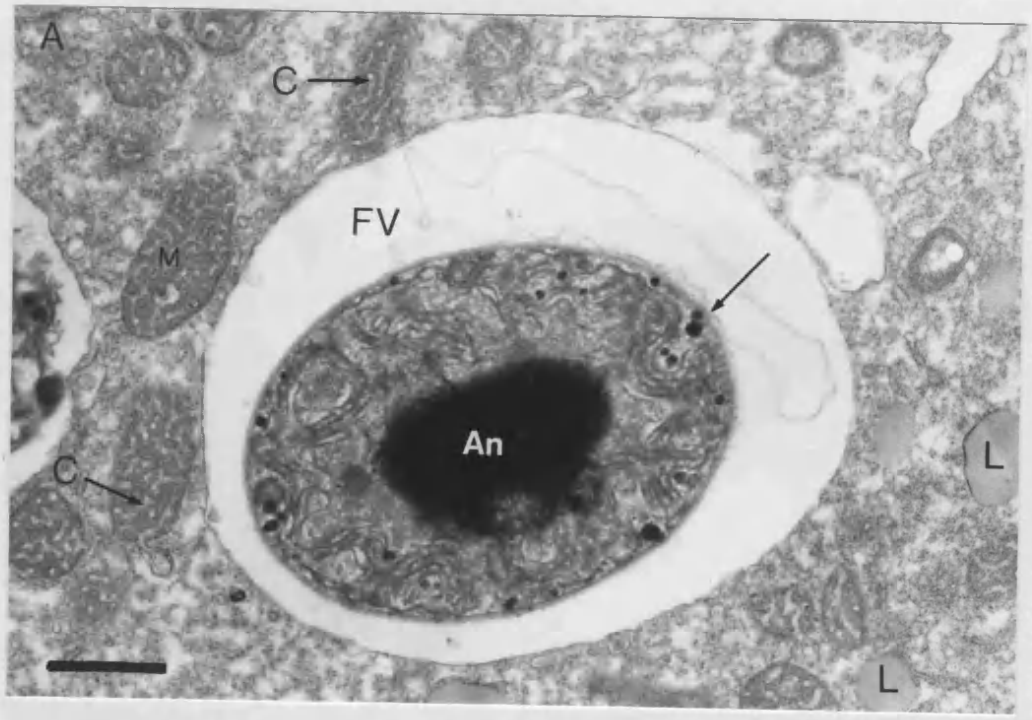


Fig. 6.3



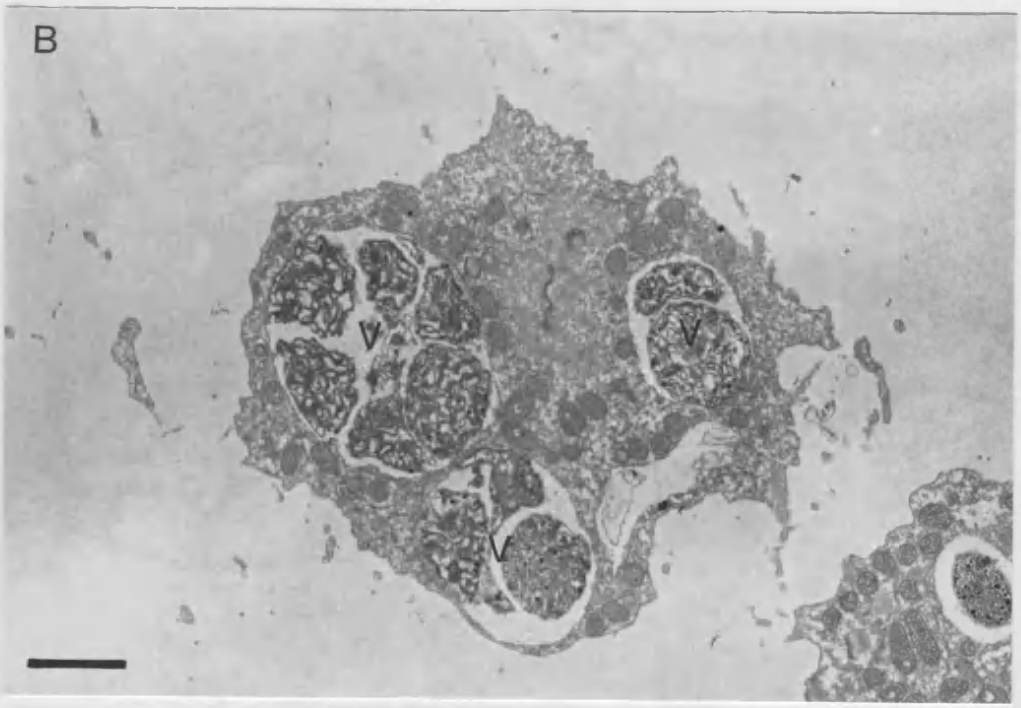
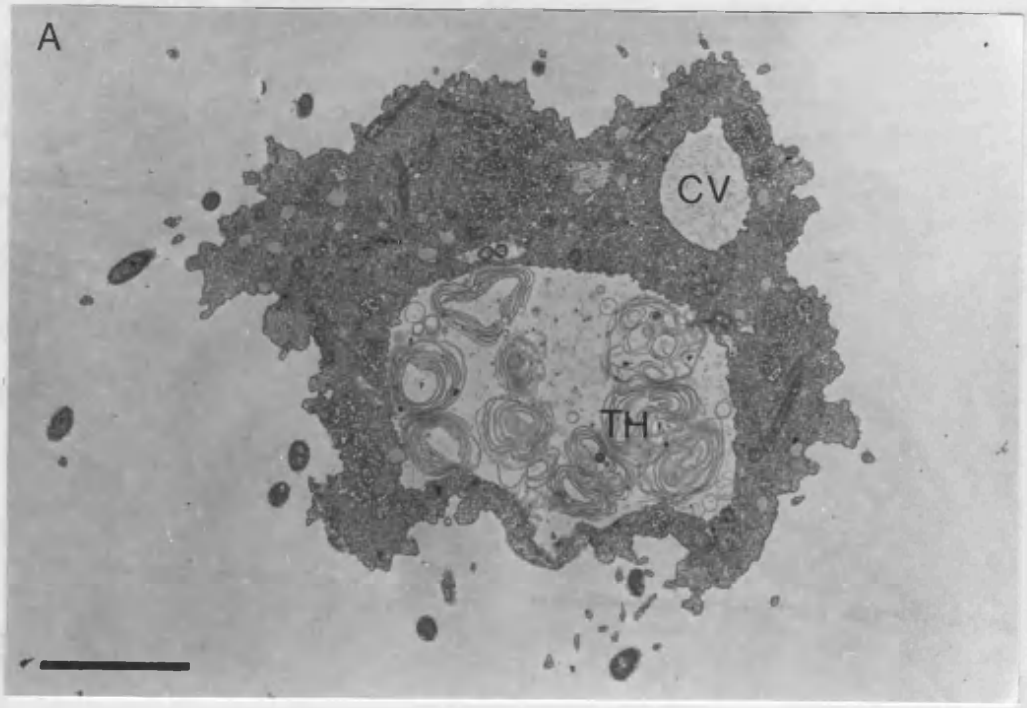
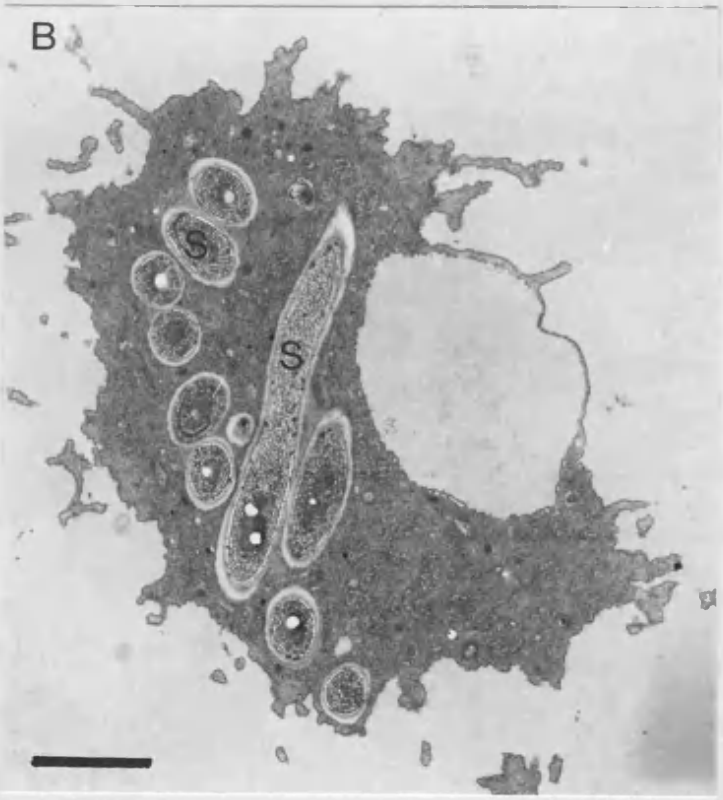


Fig. 6.4





with the plasmalemma. The acanthopodia were supported by bundles of microfibrils oriented along their longitudinal axis. Amoebae contained a single large nucleus, bound by a double membrane, with a central, dark staining nucleolus (Fig.6.1 A). Mitochondria (with tubular, branched cristae) and lipid droplets were distributed throughout the amoebal cytoplasm (Fig.6.3 A). Most cells contained vacuoles, although the size and number of these varied considerably. Each cell also contained a contractile vacuole surrounded by a tubular spongiome (Fig.6.4 A).

(2) Observations of feeding trophozoites

Trophozoites of A. castellanii PB which were fixed after 4h incubation with Anabaena A4 at 20°C almost all contained large numbers of ingested cyanobacteria. The Anabaena cells were enclosed in discrete food vacuoles, and so were effectively kept separate from the amoebal cytoplasm. Individual trophozoites often had several food vacuoles containing Anabaena cells at different stages of digestion. Fig.6.1 B shows a trophozoite with a length of newly ingested Anabaena filament (the cytoplasmic connection between two of the cells is clearly seen in Fig.6.2 A). The same cell also has food vacuoles containing cyanobacteria at early and advanced stages of digestion. With newly ingested prey there was usually a considerable gap between the cyanobacterial cell wall and the food vacuole membrane. There were no detectable morphological differences between recently internalized Anabaena cells and non-phagocytosed cells in the surrounding medium (compare Fig.6.2 A and Fig.6.2 B). Such cells were surrounded by a thick wall. The most obvious features in their cytoplasm were the

large number of tripartite thylakoids. Cells also contained small, rounded, deeply-staining granules which occurred at greatest density at the periphery of the cells (Fig.6.2 B).

The first indication of degradation of ingested cyanobacteria was destruction of the cell wall layers. Fig.6.3 A shows an Anabaena cell in which part of the cell wall is disrupted. Later in digestion the wall is disrupted over the entire surface of the cell, and the thylakoid membranes and other features of internal organization become less and less distinct (Fig.6.1 B, V2). In advanced stages of digestion the cellular integrity of cyanobacteria is completely lost. The contents of old food vacuoles consisted of dispersed thylakoid membranes (Fig.6.3 B).

Digestion of Plectonema boryanum and Synechococcus leopoliensis (Fig.6.4) was similar to that described above for Anabaena A4. With Synechococcus, cells were individually enclosed in food vacuoles (Fig.6.4 B)

Discussion

The ultrastructural organization of the Acanthamoeba castellanii strains investigated in this study (Neff G and PB) was in close accordance with that described for A. castellanii Neff by Bowers & Korn (1968). Likewise, the observed morphology of the cyanobacteria closely resembled published descriptions (see Lang (1968) for a review of cyanobacterial fine structure).

The results of the present study clearly show the progressive digestion of Anabaena A4 cells within amoebal food vacuoles. In new food vacuoles the Anabaena cells (which presumably had been ingested

shortly before fixation of specimens) were morphologically indistinguishable from non-ingested cells in the surrounding medium. In old food vacuoles the cyanobacterial prey were disrupted and dispersed beyond recognition. All stages of digestion between these two extremes were observed.

The findings of this study were similar to other reports of the digestion of cyanobacteria by protozoa (e.g. Cole & Wynne, 1974, 1975; Yamamoto & Suzuki, 1984). The former authors investigated the predation of Microcystis by the flagellate Ochromonas. As in the present study, old food vacuoles contained dispersed membrane fragments.

The presence of a distinct space separating the amoebal food vacuole membrane and the cell wall of newly ingested cyanobacteria may be an artefact caused by the differential shrinkage/expansion of the amoeba and the cyanobacterium during the preparation of specimens for electron microscopy. This suggestion is supported by the observation that the shape described by the food vacuole membrane is the same (though larger) as that of the Anabaena cell it surrounds.

It is probable that the degradation of the cyanobacterial prey of Acanthamoeba is brought about by a mechanism similar to that employed by other protozoa which feed by phagocytosis (i.e. by the release of hydrolytic enzymes into the food vacuole from lysosomes - see Nisbet, 1984). The observation that cell wall disruption appears to be an integral part of digestion may suggest a role for lysozyme in degrading the peptidoglycan layer of the cyanobacterial cell wall (see Section five).

Two ways in which these studies of intracellular digestion of

cyanobacteria by Acanthamoeba could be extended and improved are immediately apparent. Firstly, techniques could be used for the cytochemical localization of hydrolytic enzymes within amoebal trophozoites. Ryter & Bowers (1976) have already applied such techniques to study the localization of acid phosphatase in A. castellanii during growth and after phagocytosis. Secondly, it would be advantageous to fix amoebae at a series of times after introduction of the cyanobacterial food source. This would enable more critical investigation of the time scale over which digestion operates.

SECTION FIVE

ENZYME STUDIES

Introduction

If protozoa are effectively to utilize cyanobacteria as a food source, an essential requirement is that they should have the ability to disrupt and digest the prey cells. In this case the major physical barrier to the digestive enzymes of predators, apart from the mucilaginous sheath (if present), is the cyanobacterial cell wall. This is similar in structure and composition to the wall of Gram-negative bacteria, consisting of an outer membrane containing lipopolysaccharide and an inner peptidoglycan layer (Drews & Weckesser, 1982). Peptidoglycan is a heteropolymer of two amino sugars, N-acetylglucosamine and N-acetylmuramic acid, together with the amino acids alanine, glutamic acid and diaminopimelic acid. It is this layer which must initially be broken down by the predator in order to release the cell's cytoplasmic contents for digestion and assimilation. Lysozyme (N-acetyl-β glucosaminidase) is the enzyme responsible for degrading peptidoglycan, attacking the glycosidic bond between the C-1 of N-acetylmuramic acid and the C-4 of N-acetlyglucosamine (Jolles & Jolles, 1984).

In view of the probable importance of lysozyme activity in the predation of cyanobacteria by protozoa, some experiments were conducted on the enzyme activity of Acanthamoeba. Several Acanthamoeba strains were screened for their activity against a range of enzyme substrates, including that for lysozyme. Crude enzyme extracts

prepared from amoebal cultures were assayed for lysozyme activity by testing their ability to lyse cell suspensions of Micrococcus lysodeikticus. The effect of crude enzyme preparations on suspensions of cyanobacterial cells was also investigated.

Materials and Methods

(1) API ZYM strips

The enzyme activities of ten Acanthamoeba strains against 19 substrates were examined using the API ZYM System (API System S.A., La Balme, Les Grottes, 38390 Montalieu, Vercieu, France.).

Preparation of specimens

To prepare whole cell suspensions, samples (4ml) of amoebae from log-phase batch cultures were diluted with sterile PGY medium to give an OD_{400} of 0.1, which from previous calibration was equivalent to an amoebal density of $1x10^5$ cells/ml. Amoebae from these diluted suspensions were harvested by centrifugation (10min, 500g), and the supernatant was replaced by an equal volume of sterile distilled water. Suspensions of disrupted amoebae were prepared in the same way, except that cells were resuspended in 1% (v/v) Triton-X100 instead of distilled water. The Triton-X100 acted as a surfactant, causing lysis of amoebal trophozoites. Suspensions of both whole and disrupted cells were used immediately after being prepared.

Assessment of enzyme activity

Each API ZYM gallery comprised 20 cupules, 19 containing the different enzyme substrates and buffers, and one control. Two drops

(65µl) from a Pasteur pipette containing the specimen to be tested (suspension of whole or lysed cells) were inoculated into each cupule of the gallery. Each gallery was placed in a plastic tray (into which was dispensed 5ml of water to maintain a humid atmosphere) and incubated at 37°C for 4h. Following incubation, one drop of API ZYM reagents A and B was added to each cupule and the colour allowed to develop for 5min. Galleries were placed under a bright light for 10s to neutralise any colour change that had occurred in negative reactions. Enzyme activities were recorded on a scale 0 to 5 corresponding to the intensity of colour developed by reference to a colour chart prepared by the manufacturers.

One gallery was inoculated for each of the ten Acanthamoeba strains. Control galleries were inoculated with sterile PGY medium and 1% Triton-X100. In addition, some galleries were inoculated with the supernatant from centrifuged samples of amoebal batch cultures.

(2) Crude enzyme extracts

Preparation of enzyme extracts

Samples (5ml) from a 2d batch culture of Acanthamoeba castellanii PB were centrifuged for 10 min at 500g. The supernatant was discarded and the cells washed by resuspension in 5ml 0.05M citric acid/sodium citrate buffer, pH 5.0 (see Appendix B for composition). Samples were re-centrifuged and suspended in fresh buffer. Cells were disrupted by three-fold freezing (-20°C) and thawing (room temperature). After the final thawing samples were centrifuged for 10 min at 2900g. Supernatants were transferred to fresh tubes and stored at 4°C until use.

Assessment of bacteriolytic activity

Bacteriolytic activity was determined by measuring the decrease in optical density of Micrococcus lysodeikticus suspensions when added to samples of amoebal enzyme extract. Enzyme extracts were diluted by one half with 0.05M citric acid/sodium citrate buffer, pH 5.0. Samples (3.8ml) of diluted extract were transferred to 1cm path length polystyrene cuvettes (4.5ml capacity). A suspension of M. lysodeikticus was prepared by resuspending 0.01g of lyophilized cells (Sigma Chemical Company Ltd.) in 3ml distilled water. The density of this suspension was such that when 0.2ml was added to the amoebal extract in the cuvette the initial optical density (read at 550nm using a Pye-Unicam SP6-550 UV/VIS spectrophotometer) was between 0.5 and 0.6. The optical density was recorded 30 seconds after mixing and at one-minute intervals thereafter. Distilled water was used to zero the spectrophotometer. Control cuvettes contained 0.2ml of bacterial suspension with 3.8ml of buffer (minus amoebal extract). Experiments were conducted at room temperature (28°C).

To investigate the effect of buffer pH on bacteriolytic activity, enzyme extracts were prepared in 0.05M citric acid /sodium citrate buffer at 13 pH values; 3.0, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.4, 5.8 and 6.2. Bacteriolytic activity was assessed by the method described above. Control cuvettes contained 3.8ml buffer of the appropriate pH and 0.2ml bacterial suspension. The experiments were conducted at room temperature (25°C).

To determine the effect of buffer molarity on bacteriolytic activity, enzyme extracts were prepared in citric acid/sodium citrate

buffer, pH 3.6, at four molarities ; 0.1M, 0.075M, 0.05M and 0.025M. Each extract was diluted 1+4 with buffer of the appropriate molarity prior to testing for bacteriolytic activity. Duplicate experiments were run for each molarity. Experiments were conducted at room temperature (24°C).

Assessment of cyanobacteriolytic activity

Cells of Synechococcus leopoliensis 1405/1 from a 14d batch culture were resuspended in 0.05M citric acid/sodium citrate buffer, pH 4.2. The density of the suspension was adjusted such that 2ml added to 2ml amoebal enzyme extract (prepared as above in buffer at pH 4.2) in a cuvette gave an initial OD_{630} of 0.5. The optical density of the suspension was recorded one minute after mixing and subsequently at one minute intervals. Distilled water was used to zero the spectrophotometer. Control cuvettes contained buffer in place of amoebal enzyme extract.

Effects on cyanobacterial morphology

Tests were made of the activity of amoebal enzyme extract against eleven cyanobacterial cultures; Anabaena A4, A. variabilis, A. cylindrica, A. catenula, Aphanizomenon flos-aquae, Anacystis nidulans, Synechococcus leopoliensis, Plectonema boryanum, Nostoc muscorum, N. calcicola, and Calothrix parietina. For each cyanobacterium, 1ml from a 14d batch culture was dispensed into each of five Repli dish wells. Each well then received 1ml of one of four different solutions.

(a) Acanthamoeba enzyme extract in 0.05M citric acid/sodium citrate buffer, pH 4.2 (two wells for each cyanobacterium)

(b) Enzyme extract diluted 1+5 with the same buffer.

(c) 0.05M citric acid/sodium citrate buffer, pH 4.2.

(d) Allen's medium.

Those wells to which buffer or Allen's medium was added (c and d) acted as controls. Dishes were incubated in the light at 30°C for 120h. Samples were periodically removed for microscopic observation.

To investigate the effect of pH on cyanobacteriolysis, cyanobacterial/enzyme extract mixtures were prepared as above in 0.05M buffer at pH 3.6, 4.2, 4.8 and 5.4, and incubated at 30°C.

Results

API ZYM strips

The enzyme activity (against 19 substrates) of lysates prepared from 10 Acanthamoeba strains is shown in Table 7.1. To enable comparison of strains all lysates were prepared from amoebal suspensions with a density of $1x10^5$ cells/ml. The pattern of reaction displayed by each strain was very similar. Enzymes for which positive reactions were recorded could be placed into three groups based on the intensity of reaction produced. They are listed below in order of decreasing activity.

(i) Acid phosphatase, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase.

(ii) Esterase lipase, leucine arylamidase, N-acetyl-β glucosaminidase (lysozyme).

(iii) Esterase, lipase.

Lysates of most of the Acanthamoeba strains contained

Table 7.1. The enzyme activity* of crude extracts from ten strains of *Acanthamoeba* against 19 substrates.

Enzyme assayed for	*Acanthamoeba* strain									
	CCAP 1501/1a	CCAP 1501/2a	CCAP 1534/2	CCAP 1534/3	NEFF G	BATH 31	BATH PB	CCAP 1501/3a	CCAP 1501/3b	CCAP 1547/1
Control	0	0	0	0	0	0	0	0	0	0
Phosphatase, alkaline	3	5	5	5	5	5	5	5	3	5
Esterase (C4)	0	1	1	1	1	1	1	1	1	1
Esterase lipase (C8)	1	2	3	2	2	2	2	2	0	2
Lipase (C14)	0	1	1	0	0	0	1	0	0	0
Leucine arylamidase	0	2	2	2	1	1	2	2	1	4
Valine arylamidase	0	0	0	0	0	0	0	0	0	0
Cystine arylamidase	0	0	0	1	0	0	0	0	0	0
Trypsin	0	0	0	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0	0
Phosphatase, acid	4	5	5	5	5	5	5	5	5	5
Naphthol-AS-BI-phosphohydrolase	2	5	5	4	4	4	5	5	5	5
∝ galactosidase	0	0	0	0	0	0	0	0	0	0
β galactosidase	0	0	0	0	0	0	0	0	0	0
β glucuronidase	0	0	0	0	0	0	0	0	0	0
∝ glucosidase	0	0	0	0	0	0	0	0	0	0
β glucosidase	0	0	0	0	0	0	0	0	0	0
N-acetyl-β glucosaminidase	1	2	1	2	1	1	2	1	0	5
∝ mannosidase	0	0	0	0	0	0	0	0	0	0
∝ fucosidase	0	0	0	0	0	0	0	0	0	0

* Enzyme activity is expressed on a semi-quantitative scale of 0-5 corresponding to the number of nanomoles of substrate hydrolysed:

0=0 nmoles 2=10 nmoles 4=30 nmoles

1=5 nmoles 3=20 nmoles 5≥40 nmoles

sufficient of the enzymes in the first group to hydrolyse 30 nanomoles or more of substrate. Inoculation of galleries with lysates prepared from amoebal suspensions of higher cell density, for example $8x10^5$ cells/ml, resulted in positive reactions being recorded for a greater range of the enzymes assayed for. However, in these cases the test was less sensitive in determining the relative abundance of enzymes since maximal reaction (colour change) occurred in too many of the cupules. API ZYM galleries inoculated with Triton-X100 gave negative reactions for all substrates.

In galleries inoculated with whole cell suspensions of Acanthamoeba castellanii PB ($1x10^5$ cells/ml) a lower level of enzyme activity was found than in galleries inoculated with a similar suspension of lysed cells. Whereas only five nanomoles of substrate were hydrolysed by alkaline phosphatase in the whole cell suspension, forty or more nanomoles were hydrolysed in the lysed cell inoculum. Similar differences were recorded for the other main enzymes-acid phosphatase, lysozyme and napthol-AS-BI-phosphohydrolase. Two of the less abundant enzymes (esterase and leucine arylamidase) exhibited higher activity (10 nanomoles of substrate hydrolysed) in the whole cell inoculum than in that of lysed cells (5 nanomoles of substrate hydrolysed).

Galleries inoculated with the supernatant from centrifuged samples of a 2d batch culture of Acanthamoeba castellanii PB (cell density $3.8x10^6$ cells/ml) were positive for all enzymes with the exception of trypsin, chymotrypsin and ∝fucosidase. The most abundant enzymes were acid and alkaline phosphatase, napthol-AS-BI-phosphohydrolase, leucine arylamidase, N-acetyl-β

glucosaminidase and β-glucoronidase. Control galleries inoculated with sterile PGY medium gave no positive reaction.

Bacteriolytic activity

Acanthamoeba castellanii PB enzyme extract prepared in 0.05M citric acid/sodium citrate buffer, pH 5.0, exhibited strong bacteriolytic activity. In initial experiments conducted at 28°C the optical density of Micrococcus lysodeikticus suspensions decreased by 22% over a ten minute period. Further clearing occurred with extended incubation, optical density decreasing by 62% after 30 min and by 78% after an hour. There was no change in the optical density of control cuvettes.

The effect of buffer pH

The effect of buffer pH on the bacteriolytic activity of amoebal enzyme activity in the ten minute period following addition of substrate is shown in Fig.7.1. The data are representative of several repeat experiments. To aid clarity data for only eight of the thirteen pH values have been plotted. Bacteriolytic activity was greatest at acidic pH (pH 3.0), declining with successive decreases in acidity. At pH 3.0 the OD_{550} of M. lysodeikticus suspensions decreased by 44% after 10 minutes incubation, compared with 18% for pH 4.6 and 6.0% for pH 5.0. At pH 5.4, 5.8 and 6.2 there was no lysis of bacterial cells, the optical density of suspensions increasing slightly at the two highest pH values. In control cuvettes (no amoebal extract) there was no decrease in optical density at any of the pH values tested. The pattern of bacteriolytic activity shown in Fig.7.1 was not reflected

Fig.7.1. The effect of buffer pH on the rate of lysis of Micrococcus lysodeikticus suspensions by Acanthamoeba castellanii PB enzyme extract.

(0.05M citric acid / sodium citrate buffer, 25°C).

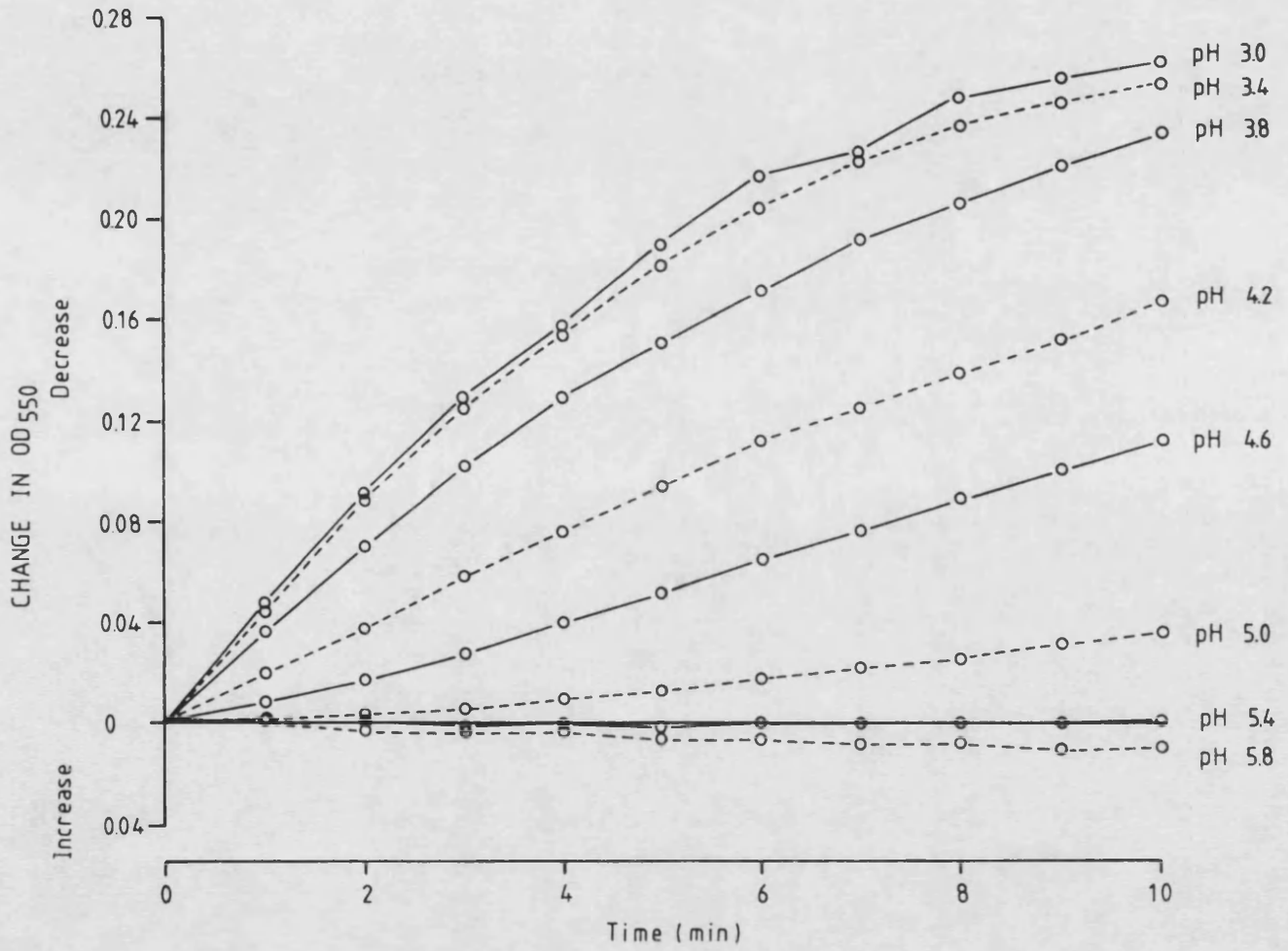
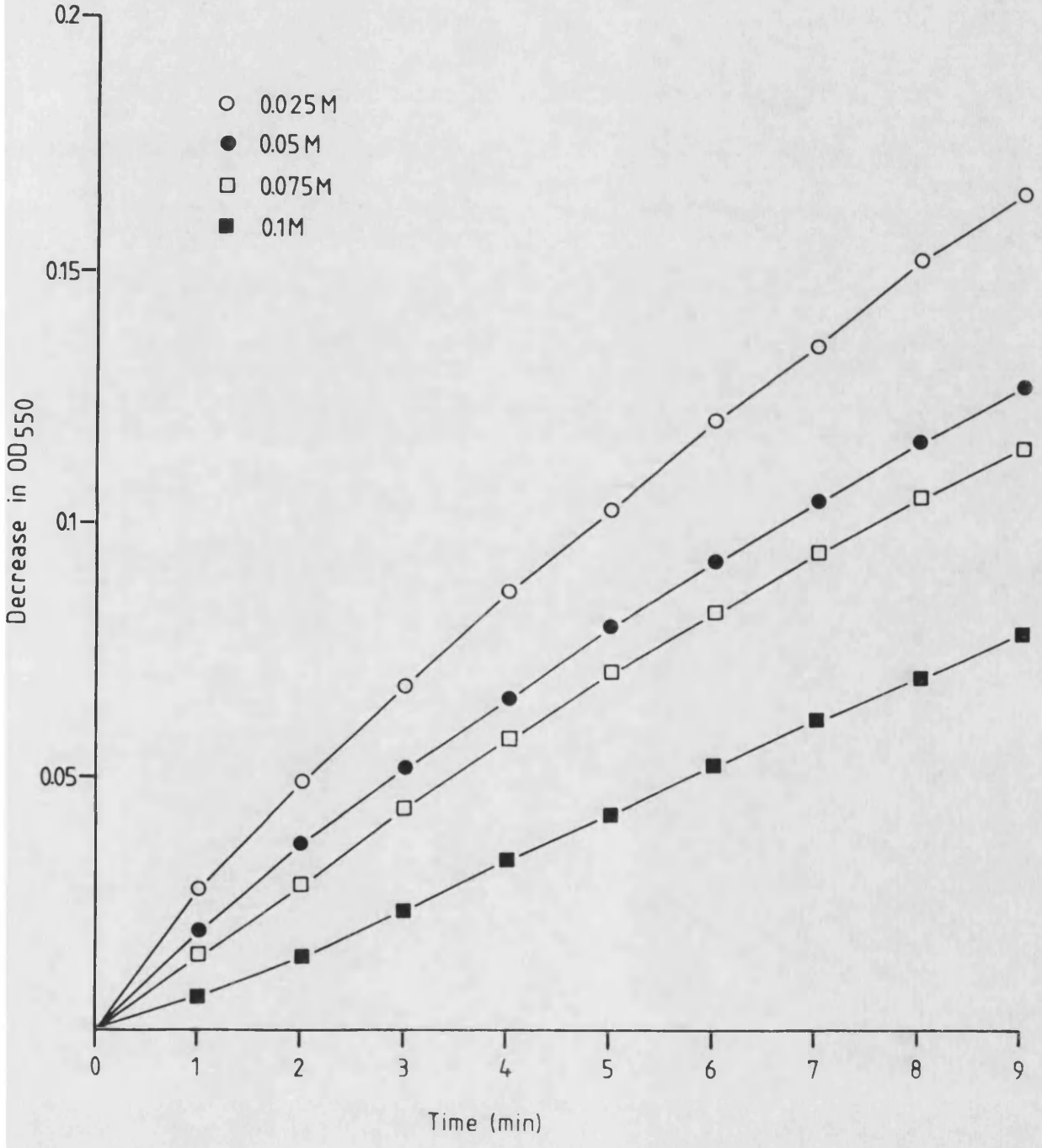


Fig.7.2. The effect of buffer molarity on the rate of lysis of Micrococcus lysodeikticus suspensions by Acanthamoeba castellanii enzyme extract.

(Citric acid / sodium citrate buffer, pH 3.6. 24°C).

Note: Values plotted are decreases in the optical density of M. lysodeikticus / enzyme extract mixtures.



in the extent of lysis achieved after extended incubation. After one hour the decrease in OD_{550} was greatest at pH 4.4 and 4.6 (73% and 74% reduction respectively) compared with decreases of just over 50% for the three lowest pH values. Over the same period the OD_{550} of controls (pH buffered, no amoebal extract) increased by between 0.8 and 2.9%. This was probably as a result of slight accumulation of bacterial cells (due to settling) in the region of the cuvette through which the spectrophotometer beam passed.

The effect of buffer molarity

The effect of varying the buffer molarity whilst maintaining pH at the same level (pH 3.6) on the bacteriolytic activity of amoebal enzyme extract is shown in Fig.7.2. Values plotted are the mean of duplicate experiments. Within the range of molarity tested (0.025 to 0.1M) lysis of bacterial cells increased as the buffer molarity was reduced. Over the initial nine minutes of incubation the optical density of M. lysodeikticus suspensions decreased by 30% in 0.025M buffer compared with 15% for 0.1M buffer. The decrease in optical density was similar at the two intermediate molarities (22% and 24% for 0.075M and 0.05M respectively). There was no change in the OD_{550} of control cuvettes (pH 3.6, no amoebal extract).

Cyanobacteriolytic activity

When crude amoebal enzyme extract was added to suspensions of Synechococcus leopoliensis there was no measurable change in optical density over the duration of the experiment (30 min) in either the experimental or control cuvettes. Rapid lytic activity of the type

observed with M. lysodeikticus suspensions did not occur.

Although short-term effects were absent, extended incubation (e.g. 20h) of mixtures of cyanobacteria and amoebal extract in Repli dish wells resulted in severe disruption of cyanobacterial cells. Of the 11 cyanobacterial strains tested, Anabaena A4 was among the most rapidly and extensively damaged. In control wells (amoebal extract replaced by buffer or Allen's medium) the Anabaena formed long filaments of regular, rounded cells, which appeared refractile when viewed under phase contrast optics. There was no alteration in morphology over the entire period of incubation (120h). By contrast, wells to which undiluted enzyme extract was added exhibited severe disruption of cells after 20h (Fig.7.3). Trichomes were fragmented into segments each of a few cells in length. Individual cells were irregular in outline, shrunken, and had a markedly granular cytoplasm. When viewed under phase contrast they were no longer refractile. The few filaments remaining intact were contorted, often coiling back on themselves. A small proportion of cells (10%) appeared unaffected by the presence of amoebal extract. These effects on cyanobacterial morphology were also evident at earlier stages of incubation (2h, 3h, 6h), although developed to a much lesser extent. In wells containing diluted (1+5) amoebal extract the disruption to cyanobacterial cells was correspondingly reduced.

The effects of amoebal extract on the morphology of other cyanobacterial strains were very similar to those described for Anabaena A4, although differences occurred in the rate and extent of disruption. As with Anabaena A4, A. variabilis, Plectonema boryanum (Fig.7.3) and Aphanizomenon flos-aquae all showed signs of damage

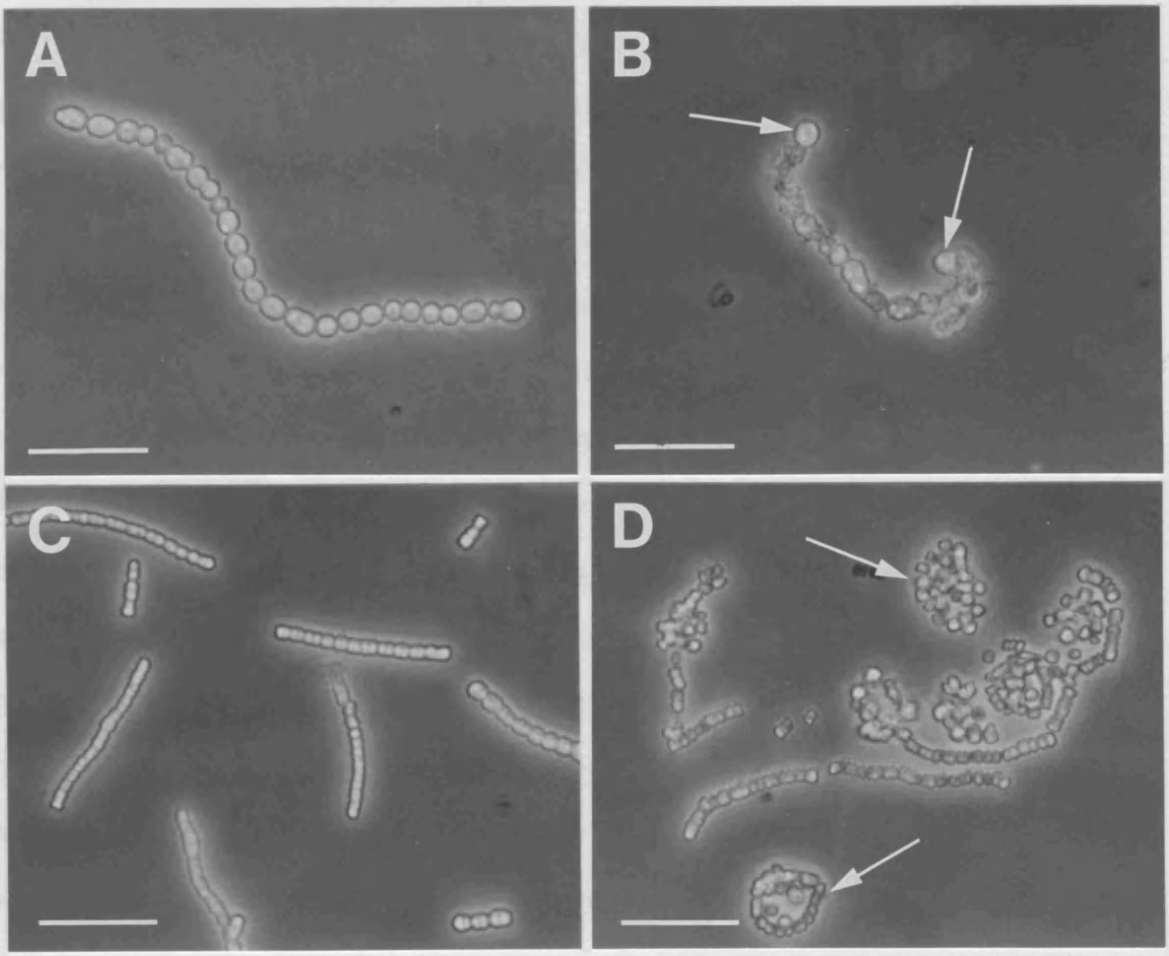


Fig.7.3. The effect of Acanthamoeba castellanii PB enzyme extract on cyanobacterial morphology.

Cells of Anabaena A4 and Plectonema boryanum were incubated (20h at 30°C) with A. castellanii enzyme extract prepared in 0.05M citric acid /sodium citrate buffer, pH 4.2. Control cells were incubated in buffer minus enzyme extract.

(A) Control cells of Anabaena A4. The filament is composed of regular, rounded, refractile cells.

(B) Anabaena cells treated with enzyme extract. Most cells show marked signs of disruption, including loss of cell shape and refractility. A few cells (arrowed) appear unaffected.

(C) Control cells of Plectonema boryanum.

(D) P. boryanum cells treated with enzyme extract. In many cases filaments have fragmented to form rounded aggregates of cells (arrowed).

(All bar markers = 20μm).

after 3h, which further developed after 20h. Trichomes of Aphanizomenon 20 or more cells in length were broken into short segments within three hours. Drops of fluid (appearing black under phase contrast) were present at many of the breakage points. The component cells of the shortened filaments were partially detached from one another, so that the filaments described a zig-zag shape as opposed to their normal straight alignment. By 20h the longest filaments contained only three cells. Individual cells had a markedly undulating outline. Anabaena catenula cells were unaffected after 6h, but extensively disrupted after 20h, whereas Nostoc muscorum and Anabaena cylindrica were damaged after 120h but not after 20h. Anacystis nidulans, Synechococcus leopoliensis, Calothrix parietina and Nostoc calcicola were the strains least affected by amoebal extract, showing only minor disruption after 120h. Growth of cyanobacteria occurred in those control wells which received Allen's medium, but not in those which received buffer. There was no morphological perturbation of cyanobacteria in either of these control treatments.

Suspensions of Anabaena A4 and Plectonema boryanum added to amoebal enzyme extract prepared at four pH values (3.6, 4.2, 4.8, 5.4) exhibited greater cell disruption with decreasing acidity.

Discussion

The API ZYM system provided a useful technique for determining the presence and relative activities of a wide range of enzymes in biochemically complex samples - lysates of Acanthamoeba cells. The presence of N-acetyl-β glucosaminidase (lysozyme) was clearly

demonstrated in all but one of the Acanthamoeba strains screened (A. polyphaga CCAP 1501/3b). This strain also exhibited less activity (relative to the other strains) for some of the other enzymes assayed for such as esterase lipase and alkaline phosphatase. This may be because the lysate inoculated into the ZYM strip was for some reason less dense than that used for the remaining strains. It is probable that inoculation of galleries with a higher density of this amoeba would confirm the presence of lysozyme. With the above exceptions, both the presence and relative activities of enzymes were very similar for the ten amoebal strains. This similarity of reaction may be indicative of close taxonomic affinity between the three Acanthamoeba species represented (A. castellanii, A. polyphaga and A. palestinensis). Indeed, Costas and Griffiths (1985) have used numerical profiles, generated from the pattern of reaction of Acanthamoeba strains to API strips, to investigate taxonomic groupings within the genus.

Disintegration of amoebae by resuspension in Triton-X100 facilitated release of enzymes from the organelles and cytoplasm into the surrounding medium, so promoting reaction with the enzyme substrate in the cupules. This would explain the greater level of enzyme activity in this treatment than that found for whole cell suspensions of amoebae. In the latter, hydrolytic enzymes would be confined largely within the amoebal trophozoites (probably bound in lysosomes ready to fuse with newly ingested food vacuoles), so reducing the degree of contact between enzyme and substrate.

The positive reaction shown by the supernatant from centrifuged samples of amoebal batch culture towards most of the 19 enzymes

assayed for indicates that considerable quantities of these enzymes occur freely in the culture medium. It is probable that they are released passively due to autolysis following death of individual amoebal trophozoites and not as a result of active secretion from viable cells, since digestion of food by these amoebae is intracellular. Drozanski (1969a) attributed an increase in bacteriolytic activity of the supernatant from Acanthamoebaculture medium at the end of exponential growth to autolysis of amoebae.

The presence of lysozyme-like enzymes in Acanthamoeba castellanii PB was further confirmed by the ability of cell lysates to clear suspensions of lyophilized Micrococcus lysodeikticus cells. A number of other workers have reported the presence of such enzymes in crude or partially purified extracts of Acanthamoeba using similar techniques (Drozanski, 1969a, 1969b, 1972; Muller, 1969; Rosenthal et al., 1969). The latter authors found that A. castellanii enzyme extract cleared suspensions of cell walls of nine species of Gram-positive bacteria, including M. lysodeikticus. Drozanski (1969a) reported the optimal pH for lysis of viable M. lysodeikticus as 4.8-5.2, whilst Rosenthal et al. (1969) found lysis of cell wall preparations of the same species optimal at pH 3-4. These values agree with the results of the present study, in which bacteriolysis was most rapid at pH 3.0.

The observed decrease in bacteriolytic activity with increasing buffer molarity (Fig.7.2) is also closely in agreement with Drozanski (1969a), who recorded increasing lysis of M. lysodeikticus as buffer molarity was reduced from 0.1 to 0.02M.

Lysozyme is most effective against the cell walls of

Gram-positive bacteria, which may contain up to 90% peptidoglycan (Witholt et al., 1976). Gram-negative bacteria have more resistance since their walls have a much lower (5-10%) peptidoglycan content, and this is protected by a lipopolysaccharide outer membrane. The inability of Acanthamoeba enzyme extracts to induce rapid lysis of cyanobacteria of the type seen for M. lysodeikticus (a Gram-positive species) was not unexpected in view of the similarity of cyanobacterial walls to those of Gram-negative bacteria (Drews & Weckesser, 1982).

The longer term effects of A. castellanii crude enzyme extract on cyanobacterial morphology were not necessarily entirely caused by the action of lysozyme or lysozyme-like enzymes. Other chemical constituents of the lysate, acting individually or in concert, may have produced the observed disruption of cells. However, the nature of the damage closely resembled the effects of lysozyme on cyanobacteria reported by Crespi et al. (1962), who found that exposure of Fremyella diplosiphon to 0.05% lysozyme in 0.03M sodium phosphate buffer (pH 6.8-6.9) led to extensive lysis after 4-6h at 25-28°C, and complete lysis after 24h. As in the present study, filaments were broken into smaller chains and then into single cells. The end products of lysis were circular, irregular, warty fragments. This similarity of effect provides strong evidence that a lysozyme-like component of the amoebal lysate is responsible for the disruption of cyanobacteria. Once lysozyme action has rendered the cell wall permeable the cyanobacterium becomes susceptible to osmotic lysis. Other enzymes present in the lysate may then contribute to the degradation of the cells.

There were no obvious physical features accounting for the differing susceptibility of cyanobacteria to amoebal lysate, with the possible exception of sheath thickness. For example, Anabaena cylindrica and Nostoc muscorum, both of which have thick sheaths, were relatively insensitive to enzyme damage, whereas Anabaena A4 and A. variabilis, which have a less pronounced sheath, were among the most rapidly affected species. These findings give support to the suggestion of Ho & Alexander (1974) that the sheath may act as a barrier to lytic enzymes, preventing their contact with the underlying cell wall and so protecting the cyanobacterium from amoebal predation. On the other hand, Crespi et al. (1962) found that species with gelatinous sheaths were most readily attacked by lysozyme, thereby inferring that such species had a greater proportion of peptidoglycan in their cell walls. An alternative explanation would be that the sheath acts as a "sponge", concentrating the enzyme and so increasing the susceptibility of the cyanobacterium to attack. Whatever the reasons, it is probable that both sheath thickness and the peptidoglycan content of the cyanobacterial wall play a part in deciding the susceptibility of cyanobacteria to attack by lysozyme-like enzymes.

Ho & Alexander (1974) also proposed that the relative inability of Hartmannella castellanii (= A. castellanii), compared with Amoeba discoides and A. radiosa, to predate Anabaena and Anacystis may be due to the absence or low activity of lysozyme-like enzymes. This proposal is not borne out by the results of this study, which clearly demonstrates the activity of such enzymes in A. castellanii. Moreover, it has been shown that A. castellanii can be a very effective predator

of both Anabaena and Anacystis spp. (see p 71).

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APPENDICES

Appendix A: Composition of media for culture of cyanobacteria

(1) Allen's medium (Allen, 1968)

Constituent	Amount (g/l distilled water)
$NaNO_3$	1.5
K_2HPO_4	0.039
$MgSO_4.7H_2O$	0.075
Na_2CO_3	0.02
$CaCl_2$	0.027
$Na_2SiO_3.9H_2$	0.058
EDTA	0.001
Citric acid	0.006
Ferrous citrate	0.006

Microelements solution : 1ml/1

pH adjusted to 7.8 with dilute (0.1M) HCl/NaOH

Composition of microelements solution:

Constituent	Amount (g/l distilled water)
H_3BO_4	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.391
$CuSO_4.5H_2O$	0.079
$Co(NO)_2.6H_2O$	0.0494

(2) Spirulina medium

Constituent	Amount (g/l distilled water)
NaCl	1.0
$NaNO_3$	2.5
$CaCl_2$	0.04
$FeSO_4.7H_2O$	0.01
Na_2EDTA	0.08
K_2SO_4	1.0
$MgSO_4.7H_2O$	0.2
K_2HPO_4	0.5
$NaHCO_3$	16.8
Allen's microelements (see above)	1ml
Vitamin B6 solution (1mg dissolved in one litre distilled water)	1ml

Appendix B: Composition of buffers

(1) Citric acid/sodium citrate buffer

Stock solutions

(A) 0.1M citric acid (21.01g in 1000ml distilled water).

(B) 0.1M sodium citrate (29.41g in 1000ml distilled water).

pH	Citric Acid (ml)	Sodium Citrate (ml)
3.0	46.5	As required
3.4	40.0	
3.6	37.0	
3.8	35.0	
4.0	33.0	
4.2	31.5	
4.4	28.0	
4.6	25.5	
4.8	23.0	
5.0	20.5	
5.4	16.0	
5.8	11.8	
6.2	7.2	

Buffers were diluted with sterile distilled water to obtain the desired molarity.

(2) Citrate phosphate buffer

Stock solutions:

(a) 0.1M citric acid (19.21g in 1000ml distilled water).

(b) 0.2M dibasic sodium phoshate (53.65g $Na_2HPO_4.7H_2O$ in 1000ml distilled water).

xml of (a) plus yml of (b) diluted to 100ml

pH	x	y
4.0	30.7	19.3
5.0	24.3	25.7
5.6	21.0	29.0
6.0	17.9	32.1
6.6	13.6	36.4
7.0	6.5	43.6

(3) Tris buffer

Stock solutions:

(a) 0.2M tris(hydroxymethyl)aminomethane (24.2g in 1000ml distilled water).

(b) 0.2M HCl.

50ml of (a) plus xml of (b) diluted to 200ml.

pH	x
7.2	44.2
7.6	38.4
8.0	26.8
8.6	12.2
9.0	5.0